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The Interactions Of Bovine Pancreatic Ribonuclease With Cupric Ions

Clifford Lee Herzig

THE INTERACTIONS OF BOVINE PANCREATIC RIBONUCLEASE WITH CUPRIC IONS

by

Clifford Lee Herzig

Department of Biochemistry

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ABSTRACT

Four Cu(II) ions are bound firmly to bovine pancreatic ribonuclease over the pH range of ionization of the four histidyl residues. Two of the Cu(II) ions are bound at conformationally-sensitive sites. At pH 5.5, the first two ions bound are located at the conformationally-sensitive sites. At pH 7.0, the first and fourth ions are bound at the two conformationally-sensitive sites. This reversal in the binding sequence is seen as being due to the small conformational change which occurs in ribonuclease over this pH range. Cu(II) destabilizes the protein but does not change the basic features nor the mechanism of denaturation. The standard enthalpy of thermal denaturation remains unchanged in the presence of Cu(II); hence the destabilization is an entropic effect. Cu(II) is preferentially bound to the denatured protein conformation.

The complete binding of one Cu(II) to the histidyl residue at sequence position 119 inhibits the carboxymethylation at this residue by iodoacetate at pH 5.5. At pH 7.0, the binding of four Cu(II) ions contributes to a total of less than complete inhibition. The inhibition is partially due to steric effects.

One of the conformationally-sensitive binding sites for Cu(II) contains the histidyl at sequence position 119. The second probably contains the histidyl at position 12. Activity is not abolished when only one or two Cu(II) ions per molecule of ribonuclease are present in solution because of the competition between ribonuclease and nucleotide substrate for Cu(II), and competition between Cu(II) and nucleotide for the histidyl-containing binding sites on the protein. The binding of Cu(II) at histidyl residues 48 and 105 does not affect the stability of ribonuclease but it is postulated that protonation of histidyl 48 may be responsible for the variation with pH of the sequence of binding of Cu(II) at the various sites.

INTRODUCTION

It has been clear since the work of Davis and Allen (1955) that Cu(II) and Zn(II) ions exert an inhibitory effect on the enzymic activity of ribonuclease. The effects of Zn(II) were partially clarified in the later work of Ross, Mathias and Rabin (1962). Crestfield, Stein and Moore (1963c) found that Cu(II) was able to inhibit the carboxymethylation of ribonuclease by iodoacetate. The reaction, at pH 5.5, was shown by them to occur at the histidyl residues at sequence positions 12 and 119 and a close spatial arrangement of these two histidyl residues was postulated. Both of these residues are known to be essential for enzymic activity (Gundlach, Stein and Moore, 1959). This work allowed the conclusion that some Cu(II) and Zn(II) ions do bind at or near histidyl residues in the active center of ribonuclease. Breslow and Girotti (1966) showed that one Cu(II) ion binds at each of the four histidyl residues of ribonuclease at a site consisting of one imidazole and two peptide nitrogens. The results of their titration experiments appeared in the literature after similar experiments had been carried out in this work.

However, the works cited above do not allow definite knowledge of the number and sequence positions of the histidyl residues which bind metal ion to affect specific properties of ribonuclease. This is true with the exception of the effect of Cu(II) on the carboxymethylation reaction at pH 5.5. It then seemed desirable to study the interactions of the protein with metal ion in further detail. The binding of metal ion might reasonably be expected to affect several properties of ribonuclease, and each in a different manner, since a particular binding site might be involved in enzymic activity, e.g., but not in conformational stability. It was believed that different effects associated with the binding of the metal ions might be isolated if experiments were conducted at low molar ratios of metal ion to ribonuclease and if buffers and other materials capable of complexing the ions were omitted from the solutions studied. The techniques used include titration studies, thermal denaturation and denaturation by urea, and chromatographic electrophoretic and pH-stat determinations of the kinetics of carboxymethylation of ribonuclease. Most of the work was carried out using Cu(II) since it was found that it was more convenient to study Cu(II) than Zn(II).

The principal aims of this work were to determine the number of histidyl residues which bind Cu(II) to effect

i. inhibition of enzymic activity

ii. a change in the stability of the protein

iii. inhibition of carboxymethylation.

In addition, it was hoped that sequence positions could be determined for each of the histidyl residues which contribute to each of these three effects upon the binding of Cu(II). It will be shown that all of these aims were either completely or partially fulfilled. It was also found that the results of the thermal denaturation experiments over a range of pH values produced evidence on the mechanism and thermodynamics of thermal denaturation of ribonuclease, largely through the derivation of equations which relate the binding of a given amount of Cu(II) to the state of equilibrium at a given temperature and to the transition temperature. It is believed that the techniques used in this work are of general value to workers studying the interactions of metal ions with proteins. Specifically, measurement of the stability of a metal:protein complex, relative to that of the protein itself, can provide an important tool for analysis of the interaction.

II. HISTORICAL REVIEW

A. Bovine Pancreatic Ribonuclease A

1. Source and Molecular Parameters

Ribonuclease (polyribonucleotide 2-oligonucleotide-transferase (cyclizing)) (2.7.7.16) has been the subject of extensive physical and chemical investigation. Mammalian pancreas is the usual source of the protein although significant amounts have been found in other organs. It is believed that the bulk of synthesis of ribonuclease takes place in the pancreas and that the presence of the enzyme in other organs is a result of metabolic mobilization. Schucher and Hokin (1954) have demonstrated the synthesis of high levels of ribonuclease by pigeon pancreas slices under aerobic conditions when an adequate supply of amino acid mixture is ensured. Dixon and Webb (1964) provide a convenient collation, with references, of comparative levels of ribonuclease activity in various organs from selected mammalian sources.

Most of the work done in recent years has been carried out with homogeneous bovine pancreatic ribonuclease (hereafter termed RNase). This enzyme belongs to the loosely-defined solubility class of globulins; i.e., it is soluble in aqueous solution but precipitates in $(NH_4)_2SO_4$ solutions of greater than 50% saturation.

RNase has a molecular weight, by amino acid summation, of 13,700. The molar absorptivity at 217.5 mμ is 9,800, as determined by Tanford, Hauenstein and Rands (1955); Sela, Anfinsen and Harrington (1957); and Bigelow (1961). The specific optical rotation at 436 mμ is -151° at pH 6.8 (Hermans and Scherage, 1961 a).

The amino acid composition of RNase was determined by Hirs, Moore and Stein (1956); the amino acid sequence was established by Hirs, Moore and Stein (1960). Both sets of determinations were modified by Smyth, Stein and Moore (1962) and by Potts, Berger, Cooke and Anfinsen (1962). The positions of the disulfide bridges were determined by Spackman, Stein and Moore (1960).

Recently, two research groups have published preliminary three-dimensional structural models of RNase based on their X-ray diffraction studies. While the model of Avey, Boles, Carlisle, Evans, Morris, Palmer and Woolhouse (1967) differs from

that of Kartha, Bello and Harker (1967) in the location of one disulfide bridge, there are no significant differences between the models that would affect conclusions to be drawn in this thesis. Figure 1 shows the amino acid sequence of RNase as given by Kartha et al (1967); the sequence is that originally presented by Smyth et al (1962). A schematic diagram of the model of Kartha et al (1967) is shown as Figure 2.

2. Isolation and Purification

The crystallization of ribonuclease was first accomplished by Kunitz (1940), who used $(NH_4)_2SO_4$ fractionation. This method was refined by McDonald (1948 a, b) who introduced a five minute boiling step after the initial bulk precipitation by $(NH4)2SO4$ from solubilized pancrease extract. The modification was designed to free the crude mixture from protease contamination, and is included in the combined procedure of Kunitz and McDonald (1953).

Hirs (1953) has published a procedure for the isolation of RNase using the synthetic cation-exchanger, Amberlite IRC-50 (BioRex 70, Rexyn 102). Dried bovine pancreas extract is taken up in 0.2 M phosphate buffer at pH 6.03 and applied to the ion-exchange column without recourse to fractionation by $(NH_4)_2SO_4$. However, it is stated by Hirs (1953) that it is more convenient to chromatograph

FIGURE 1

The covalent structure of RNase diagram is from Kartha et al (1967).

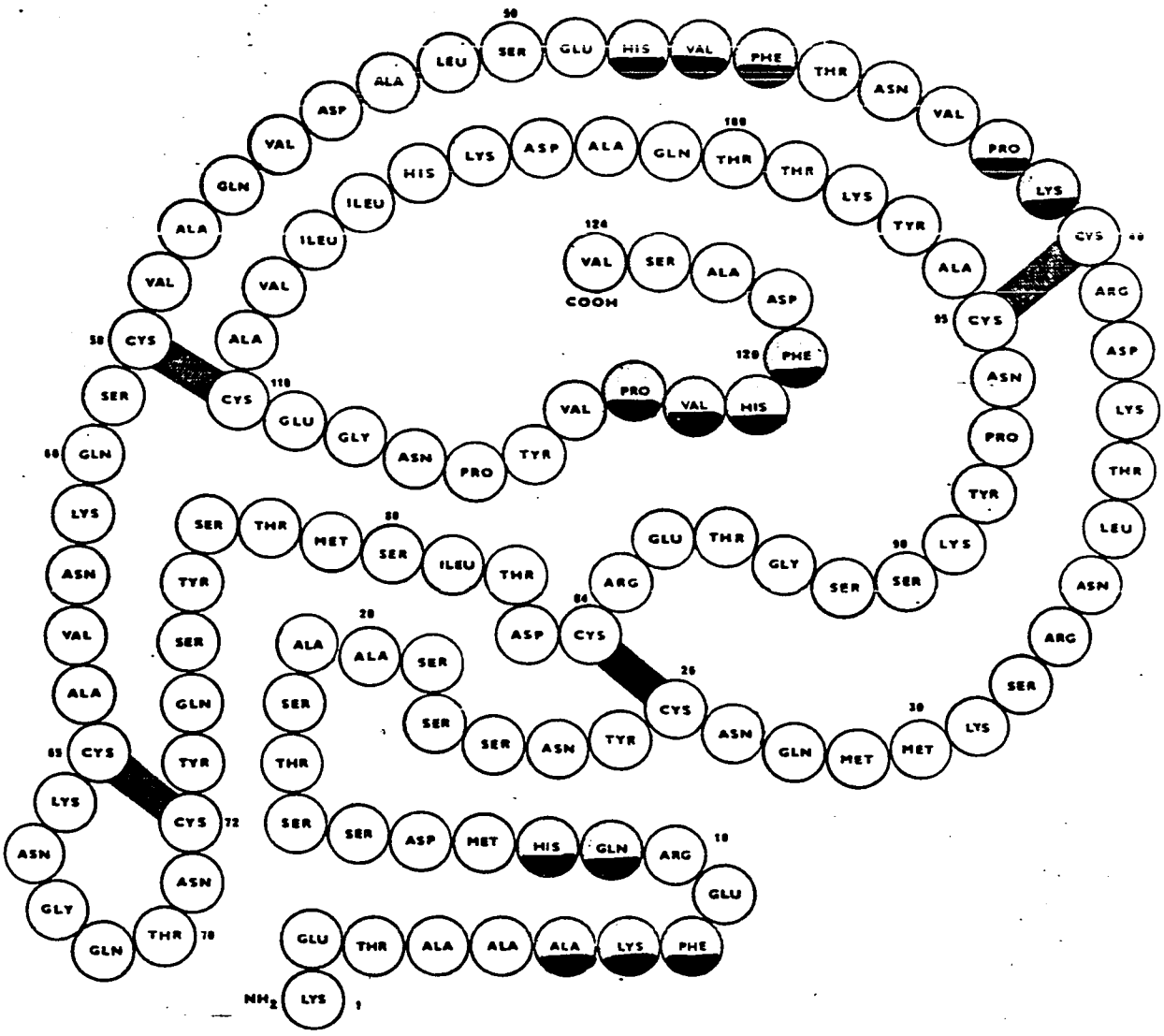
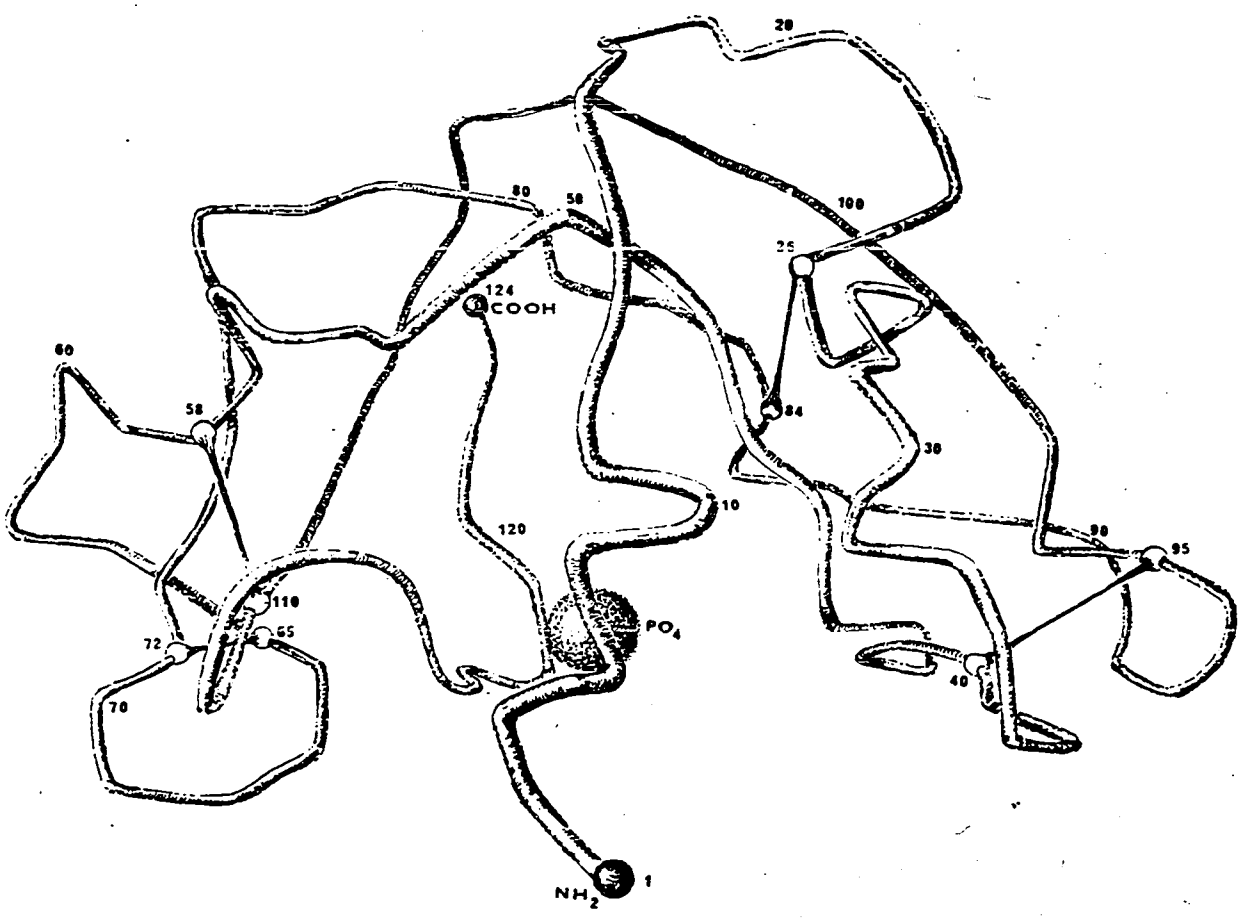

NH2 LYS 1
GLU THR ALA ALA ALA LYS PHE
GLN THR 70 GLY ASN CYS 72 LYS ASN ALA CYS 83 GLN LYS ASN VAL ALA CYS 58 SER GLN 60
SER SER ASP MET HIS GLN ARG 10 GLU
THR SER 80 MET SER ILEU THR TYR SER GLN TYR
SER SER THR ALA ALA SER SER SER SER SER
ASP CYS 26 ASN GLN MET MET 30 LYS SER ARG ASN LEU THR LYS SER SER ASN TYR
ARG GLU THR GLY SER SER 90 LYS TYR PRO ASN LYS ASP ARG CYS 95 CYS 48
ASP ALA GLN THR THR LYS TYR ALA LYS PRO VAL THR ASN VAL PHE HIS LYS ASP ALA LEU SER GLU HIS VAL PHE 50
ILEU ILEU VAL ALA CYS 110 GLU GLY ASN PRO TYR VAL PRO VAL HIS
VAL SER ALA ASP PHE 120
COOH 124

FIGURE 2

Schematic diagram of the main chain folding in the ribonuclease molecule. Diagram is from Kartha et al (1967).


20
80
58
100
25
124
COOH
60
58
84
38
10
120
98
95
110
65
72
PO_4
40
70
NH_2
1

protein prepared by the method of Kunitz and McDonald (1953). If ribonuclease, freshly prepared by the method of Kunitz and McDonald, is carried to the 5x recrystallization stage of purification, about 85% of the protein elutes from IRC-50 as the single component, RNase A (Hirs, 1953).

The most widely used preparative procedure for RNase A at the present time is the chromatographic procedure of Hirs, Moore and Stein (1953) or its modification by Crestfield, Stein and Moore (1963 a). In this method, commercial samples prepared by the method of Kunitz and McDonald (1953) (generally designated as "ribonuclease, 5x recrystallized") are chromatographed in up to 1 gm lots on IRC-50 columns at pH 6.47 in 0.2 M phosphate buffer. Depending on the commercial sample used, between 60 and 90% of the total protein load appears as RNase A. Heterogeneity in the commercial samples appears to be due to the presence of any or all of RNase B, deamidation product, aggregate, and other nucleic acid and protein components (Crestfield, et al, 1963 a).

Fractionation of the commercial 5x crystallized ribonuclease has also been carried out using carboxymethyl cellulose by Taborsky (1959) and by Åqvist and Anfinsen (1959). Although chromatographic procedures using carboxymethyl cellulose have not become popular, they provide confirmation of the results

obtained with IRC-50. Such confirmation has also been provided by King and Craig (1958) using countercurrent distribution, Raacke (1956) with starch electrophoresis, and by other workers using a variety of techniques.

3. Storage

The storage form and subsequent stability of RNase A following chromatographic purification by the method of Hirs et al (1953) is dictated principally by the requirements of the particular experiments planned. Frequently, it is permissible to freeze solutions of RNase in the phosphate buffer in which the protein emerged from the column. Crestfield et al (1963 a) have shown that solutions of RNase A in phosphate buffer can be kept at -20° for over two years without detectable change. Samples can be thawed and kept at 4°C for weeks.

Often, however, it is important to have RNase as free as possible from inorganic ions, particularly from the multivalent phosphate and sulfate ions. These ions are known to bind to RNase in such a manner as to interfere with the normal activity and denaturation behavior of the protein. Such effects have been observed by a number of workers, including Sela and Anfinsen (1957), Sela et al (1957), Barnard (1964 a, b), and Ginsburg and Carroll (1965).

It is then essential that the chromatographic column effluent containing the protein be desalted. Such desalting is best carried out by the procedure of Dixon (1959). The desalted protein must be lyophilized and stored in the dry state at -20° for maximum stability; but as discussed below, maximum stability for the desalted lyophilized protein is less than that for RNase stored frozen in phosphate buffer.

Lyophilization is carried out from 5% acetic acid (Crestfield et al, 1963 a); a procedure which has been shown (Crestfield, Stein and Moore, 1962) to result in the formation of RNase A aggregates. Such aggregates are reversible, however, and may be dissociated by heating the protein to 65° for 10 minutes before experiments are conducted. The aggregates are detectable by column chromatography; they are irreversibly bound to IRC-50 and may be detected as a fast-moving peak on Sephadex G-75. If the 5% acetic acid is omitted from the lyophilization solution, a different type of RNase A aggregate forms. This aggregate is irreversibly formed and may not be dissociated by heat.

Desalted and lyophilized RNase A will develop up to 10% aggregate in one year at 4° (Crestfield et al, 1963a). The aggregate is of the reversible form, however. Prolonged storage of the protein is also said to result in the formation of deamidation products

(Raacke, 1956; Crestfield et al, 1963a). The problem is intensified if the protein is stored as a solution.

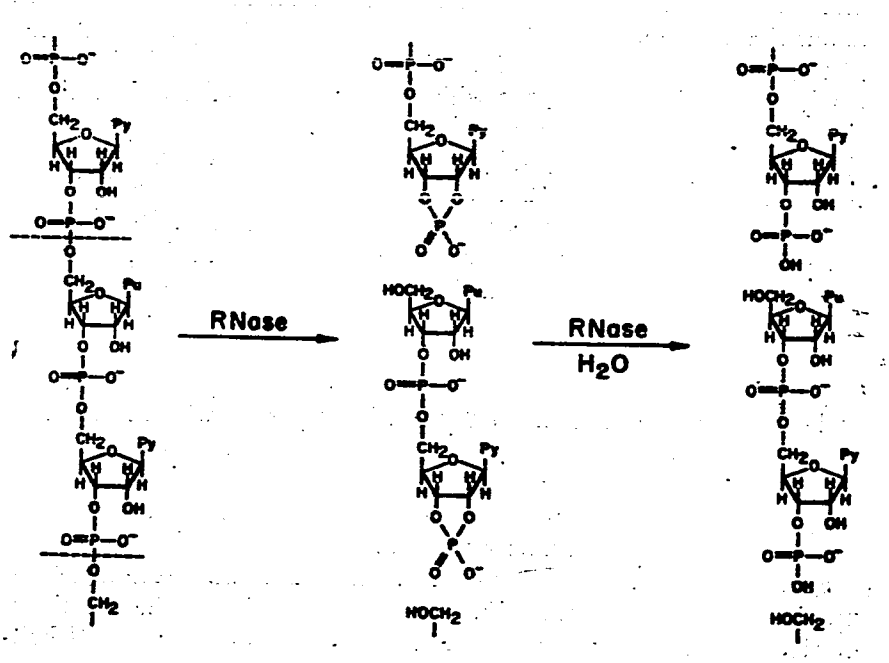
4. Activity and Assay

The action of RNase on ribonucleic acid is shown in Figure 3 (Scheraga and Rupley, 1962). The reaction consists of two stages. The first of these is a transesterification (cyclizing) reaction whereas the second stage involves the hydrolytic cleavage of the cyclic phosphoryl groups. The second stage only results in a net release of protons at near neutral pH values. In the first stage of the reaction, phosphate ester linkages appear to be cleaved only if they follow pyrimidine bases (Brown and Todd, 1955; Westheimer, 1962).

Assay procedures for RNase A may then be based on either of the two stages shown in Figure 3. There have been many assay procedures developed using several types of instrumentation; these are reviewed by Anfinsen and White (1961) and only a few representative methods are discussed here. In the first stage of the reaction, the depolymerization of ribonucleic acid results in a change in the ultraviolet absorption of the assay solution. The change is maximal at 300 mμ and was used by Kunitz (1946) as the basis of an assay procedure. His procedure is still used by many workers and most commercial ribonuclease samples have the

FIGURE 3

Action of RNase on ribonucleic acid. Pyrimidine and purine bases are represented by Py and Pu, respectively.


RNase
RNase
H_2O

activity assay results for the sample listed in terms of Kunitz units.

Since it is difficult to obtain RNA in a reproducible and homogeneous form on a routine basis, the second stage of the reaction shown in Figure 3 has been developed by a number of workers. Most recent studies designed to elucidate the nature of the active center and the mechanism of action have been carried out using either cyclic cytidine-2', 3'-phosphate or cyclic uridine-2', 3'-phosphate as substrate. Typical of the assay procedures used are the titrimetric method of Gundlach, Stein and Moore (1959) and the photometric method of Crook, Mathias and Rabin (1960). The photometric method is based on the fact that hydrolysis of the cyclic phosphate ester produces an absorption peak with a maximum at 284 mμ.

5. Active Center

Nearly all of the functional groups of RNase have been reacted with various reagents to determine which amino acid residues are essential for enzymatic activity or for conformational stability. A convenient listing of the results of these experiments, complete to 1962, is found in the article by Scherega and Rupley (1962).

Convincing evidence that a particular residue is essential for activity has been found only for the four residues histidyl 119, histidyl 12, lysyl 41 and glutaminyl 11. The evidence for considering glutaminyl 11 essential for activity comes largely from the deamidation experiments of Vithayathil and Richards (1961).

Early experiments involving photooxidation of the histidyl residues were carried out by Weil and Seibles (1955). Complete activity loss was observed following the destruction of probably only one of the histidyls, but its location in the sequence was not established. Gundlach et al (1959) and Barnard and Stein (1959) found that reaction of RNase with iodoacetate and bromacetate, respectively, resulted in an inactive carboxymethylhistidyl derivative after the uptake of one equivalent of reagent. Later experiments by Crestfield et al (1963 b, c) showed that both histidyls 119 and 12 are carboxymethylated in a concurrent reaction, but at a ratio of between 8:1 and 10:1. Even though Glick, Goren and Barnard (1967) have shown that methionine residues are also carboxymethylated in significant amounts during the reaction, methionyl substitution modification does not affect enzymic activity and only the two histidyl residues have been placed at the active center as a result of these experiments.

Klee and Richards (1957) showed that guanidation of lysyl residues by reaction with 0-methylisourea also produces an inactive RNase derivative. Hirs (1962); Hirs, Halmann and Kycia (1965) and Hirs and Kycia (1965) found that if RNase is dinitrophenylated, the loss of activity may be correlated with the disappearance of one lysyl residue, at position 41, which is unusually reactive. Largely as a result of these experiments, the lysyl residue at position 41 has been placed at the active center of RNase.

B. Protein Titration Curves

1. General

Since, in most common proteins, one out of every three or four amino acid residues contains a titratable acidic or basic group, the titration curve for a protein may be expected to appear as the summation of all the titration curves for the residue functional groups. This is true as a first approximation but various effects are known to produce deviations in the titration behavior. The most important cause of deviation is electrostatic interaction between groups which varies according to the charge on the protein and the degree of functional group dissociation at any particular pH value. As a result of this interaction, any titratable group type on a protein molecule will require a range of 3.0 or even more pH units to go

from 9% to 91% dissociation rather than the 2.0 pH units predicted by the Henderson-Hasselbach equation.

The most widely-used theory designed to take electrostatic interactions into account when analyzing protein titration curves is the "smeared site" theory proposed by Linderstrøm-Lang (1924) and developed at length by Cannan, Palmer and Kilbrick (1942) and by Tanford (1961). The theory derives its name from its basic assumption that the charge on a protein molecule is evenly distributed over the surface of a sphere which is impenetrable to solvent. According to this theory, the equation for the titration curve as a whole is

$$\bar{r} = \sum_i \frac{n_i \, K_{int}^{(i)} \, e^{2\omega \bar{Z}} / A_{H^+}}{1 + K_{int}^{(i)} \, e^{2\omega \bar{Z}} / A_{H^+}} \qquad (1)$$

where $\bar{r}$ is the number of hydrogen ions taken up or dissociated per protein molecule and $\bar{Z}$ is the average charge; both quantities apply at a given A_{H^+}. $K_{int}^{(i)}$ is the intrinsic dissociation constant for the group considered, i.e. when electrostatic interaction is absent and $\bar{Z}$ is zero. The important constant ω, the electrostatic interaction work factor, in equation 1 is the same for all titratable groups, at least in theory, and is defined as

$$\omega = \frac{\epsilon^2}{DRkT} \left(1 - \frac{\kappa}{1 + \kappa A}\right) \qquad (2)$$

where ϵ is the unit of electron charge, D is the dielectric constant of the solvent, k is Boltzmann's constant, and κ is proportional to the square root of the ionic strength. R is the radius of the protein sphere and A is the radial distance of closest approach by the average salt ion in the solution to the center of the sphere.

In practice, an equation which precedes equation 1 in the sequence of derivation is more widely used as it is an general expression for the titration behavior of any particular group type. The equation is

$$pH - \log \left[\alpha_i/(1-\alpha_i)\right] = pK_{int}^{(i)} - 0.868\,\omega\bar{Z} \qquad (3)$$

and applies to all titratable groups so long as the ionic strength remains constant. If the left-hand side of this equation is plotted vs $\bar{Z}$, pK_{int} will be the intercept and ω is obtainable from the slope. α_i is the average degree of dissociation.

Curvature in plots produced by equation 3 are usually investigated with respect to both pK_{int} and ω. Generally ω is held to be the most informative of the two parameters, partly because it can be calculated independently by equation 2 and partly because of the large number of conditions which can affect its value. Among the more probable causes for deviation in values of ω are protein denaturation, aggregation and dissociation, solvent-

inaccessible groups of type i and non-spherical molecules. Tanford (1962) gives a lucid discussion of means of evaluating many types of anomalies in experimental values of both pK_{int} and ω.

2. Titration of RNase

The first complete titration curve for RNase was produced by Tanford and Hauenstein (1956). The curve was found to be reversible between the acid end point and the onset of alkaline denaturation at pH about 11. All titratable groups found by amino acid analysis titrate in the regions expected except for three anomalous tyrosyl residues. The presence of these seemingly solvent-inaccessible tyrosyl residues was first reported by Shugar (1952) and has been confirmed by a number of workers.

The amino groups and imidazole groups of RNase showed normal pKint values and produce a value for ω which is compatible with that exhibited by the three normal tyrosyl residues. In addition to the anomalous tyrosyl residues, a minor difficulty is found in the titration of the carboxyl groups, where the expected number of carboxyl groups titrate but give too large a value for ω. Tanford and Hauenstein (1956) show that the acid part of the titration curve can be made to give the same value for ω as does the remainder of the curve if it is assumed that five of the ten side-chain carboxyl groups have a pK_{int} of 4.0 while five others have a pKint of 4.7. Hermans

and Scheraga (1961 b) achieve a similar result assuming one pK_{int} of 2.5, another of 3.7 and eight others of 4.6. Although Tanford and Hauenstein (1956) point out that the apparent anomalous behavior of the carboxyl groups could be a result of inadequacy of the theory, the results of methylation experiments carried out by Broomfield, Riehm and Scheraga (1965) indicate that there are, in fact, three carboxyl residues of abnormally low reactivity. These were assumed to be involved in tyrosyl-carboxylate interactions.

Bull and Breese (1956) offer evidence that at ionic strengths of 0.15 or greater, the smeared site theory does not apply to RNase. Using a less involved theory covering one titratable group type at a time, these authors have arrived at yet another set of values of pK_{int} for the carboxyl and imidazole groups. According to their data, no less than seven carboxyl groups could be considered to be anomalous. It is possible that the approach of Bull and Breese (1956) might yield more accurate values of pK_{int} than does the smeared site theory but it lacks a parameter offering as much valid information as is obtainable from the electrostatic work factor.

C. Metal-Binding to Proteins

1. Theory

It is observed as expected that the basic functional groups

of proteins are capable of binding metal ions in competition with hydrogen ions, although the nature of the binding site varies with pH as well as with the structure of the particular protein investigated. For this reason, the most fruitful theoretical approach to the binding of metal ions by protein has been the extension of the theory governing hydrogen ion titration curves.

Again following Tanford (1961), the equation governing the complete titration curve for the protein in the presence of metal ion may be expressed as

$$\frac{\tilde{\nu}_m}{n - \tilde{\nu}_m} = \frac{(k_{int})_m \, e^{-2\omega Z_m \bar{Z}} \, [M]}{1 + A_{H^+}/(K_{int})_{H^+} \, e^{2\omega \bar{Z}}} \qquad (4)$$

where $\tilde{\nu}_m$ is the average number of combined metal ions per protein molecule, $(k_{int})_m$ is the intrinsic association constant for metal ions of charge Z_m and n is the number of binding sites of a single type. Other terms are as previously defined.

To use equation 4, values for $\tilde{\nu}_m$ must be determined by a suitable method such as equilibrium dialysis or gel filtration and complete titration curves produced in the presence and absence of metal ion. A constancy of $(k_{int})_m$ over a range of values of pH and $\tilde{\nu}$ may be taken as proof that only one type of titratable group is involved in the binding site. The most frequently cited example of this procedure is the work of Gurd and Goodman (1952) who showed

that imidazole groups only are involved in the binding of Zn(II) to serum albumin.

Equation 4 has been extended by Tanford (1961) to cover situations where n binding sites exist, each of which has more than one of the same type of titratable group as ligands to the metal ion. However, the situation is more difficult where the metal ion is bound to a chelate site containing more than one type of ligand. In these situations the application of equations such as equation 4 would result at best in constancy of an apparent $(k_{int})_m$ over very narrow regions of the titration curve. In addition, it is frequently not possible to judge from the titration curve alone the nature of all the ligands contributing to the chelate site. But, as discussed below, the existence of chelate sites may often be inferred from the shape of the titration curves and, if adequate model studies are available for the suspected sites, confirmation of the nature of the site may be possible by curve-fitting using appropriate extensions of the theory summarized above. Fortunately, such has been the case for RNase.

2. Metal Binding to RNase A

Early results of work on the effects of metal ions on RNase activity presented a somewhat confusing picture. Mg(II), Ca(II), and Mn(II) were reported by Lamanna and Mallette (1949) to inhibit the action of RNase. However, these same ions were aaid by Zittle

(1946) and by Bain and Rusch (1944) to have no effect and Carter and Greenstein (1947) classed Mg(II) as an activator. The situation was clarified by Davis and Allen (1955) who devised a somewhat complicated but accurate assay procedure using the cyclic pyrimidine monophosphates as substrates. Product analysis was carried out by electrophoresis. Of a number of divalent metal ions tested, only Cu(II) and Zn(II) were found to be inhibitory. It is difficult to draw quantitative conclusions from the work of Davis and Allen (1955) as they added metal ion directly to assay solutions containing an excess of substrate. Since the substrate is capable of binding metal ion, the extent of such binding must be measured directly or the data must be used only in a relative sense. The work of Davis and Allen (1955) does, however, show that Cu(II) and Zn(II) are inhibitory to RNase and that Zn(II) is probably the less effective of the two ions.

Ross, Mathias and Rabin (1962) made the first involved study of the interaction between RNase A and a divalent metal ion. Zn(II) was chosen as the inhibitory ion and activity assays were carried out in the presence of metal ion and in the presence of several nucleotides known to be bound at the active center of RNase. Ultraviolet spectral data is also presented for mixtures of RNase and metal ion; some experiments included the addition of nucleotide. These authors were able to confirm the inhibitory effect of Zn(II) on

RNase activity and obtained evidence for the formation of a ternary complex between RNase, Zn(II) and the product cytidine-3'-phosphate although inhibition is not dependent upon the formation of such a complex. Unfortunately, the value determined for the association constant, K:

$$K = \frac{[Zn:Rnase]}{[Zn]\,[Rnase]} \qquad (5)$$

was probably influenced by the presence of excess substrate as well as by the formation of ternary complex. Log K was reported to be 3.9, a value which appears to be quite high when evaluated by the titration data of Breslow and Girotti (1966), and is certainly too high if only a single imidazole group is involved as a binding site (Edsall, Felsenfeld, Goodman and Gurd, 1954). However, as will be discussed below, it is difficult to interpret data concerning the nature of the binding of Zn(II) by RNase and such difficulties are probably reflected in the value of log K reported by Ross et al (1962).

Breslow and Girotti (1966) present a thorough study of the binding of both Cu(II) and Zn(II) ions by RNase, using protein titration curves and visible absorption spectra of the metal:protein complex (for Cu(II) only). Again, some measurements were made in the presence of various nucleotides. Some of the conclusions and calculations are based on model studies of histidine-containing

peptides (Bryce, Roeske and Gurd, 1965) and on the studies of Breslow and Gurd (1963) concerning the interaction of metmyoglobin with these same ions.

Figure 4 shows the data of Breslow and Girotti (1966) for the titration of RNase in the presence of $CuCl_2$. The inset in this figure shows the difference titration curve, i.e., the titration curve in the absence of Cu(II) substracted from that in the presence of the metal ion. Figure 5 shows these authors' data for Zn(II) presented as the difference titration curve only. Figure 6 shows the visible absorption spectra for various Cu(II):RNase complexes.

Two features of Figure 4 support the idea that Cu(II) binds at a chelate site in RNase. The most important of these is the fact that each of the two steps in the difference titration curve (inset) is accompanied by the release of more than one proton per metal ion bound. This clearly cannot happen if, e.g., copper ion were to bind at a site consisting only of an imidazole residue. Tentative assignment of the nature of the ligands involved is made on the basis of the pH range over which the metal ion releases protons. Thus it is most reasonable to suppose that the ligands releasing protons between pH 5 and 6 in the difference curve are imidazole groups and that the ligands involved in the more alkaline step are from a titratable group type with a pK_{int} greater than pH 10. Implicit in this

FIGURE 4

Continuous titration of RNase in the presence of 0, 1, and 2 M eq of $CuCl_2$. The conditions were: 0.16 ionic strength, 25°, an initial protein concentration of 1.0%. Without $CuCl_2$: ●——●, pH 5.5 to 3; O——O, pH 3 to 11; ⊗——⊗, pH 11 to 5.5. Molar ratio of Cu(II) to RNase of 1: ◐, pH 5.5 to 3; ◘, pH 3 to 11; ■, pH 11 to 5.5. Molar ratio of Cu(II) to RNase of 2; O, pH 5.5 to 3; Δ, pH 3 to 11; ▲, pH 11 to 5.5.

Inset:——, the difference in $\bar{r}$ between the titration curve in the absence of $CuCl_2$ and the titration curve in the presence of 1 eq of $CuCl_2$; ---, the difference in $\bar{r}$ between the titration curve in the presence of 1 eq of $CuCl_2$ and the titration curve in the presence of 2 eq of $CuCl_2$.

The data are from Breslow and Girotti (1966).

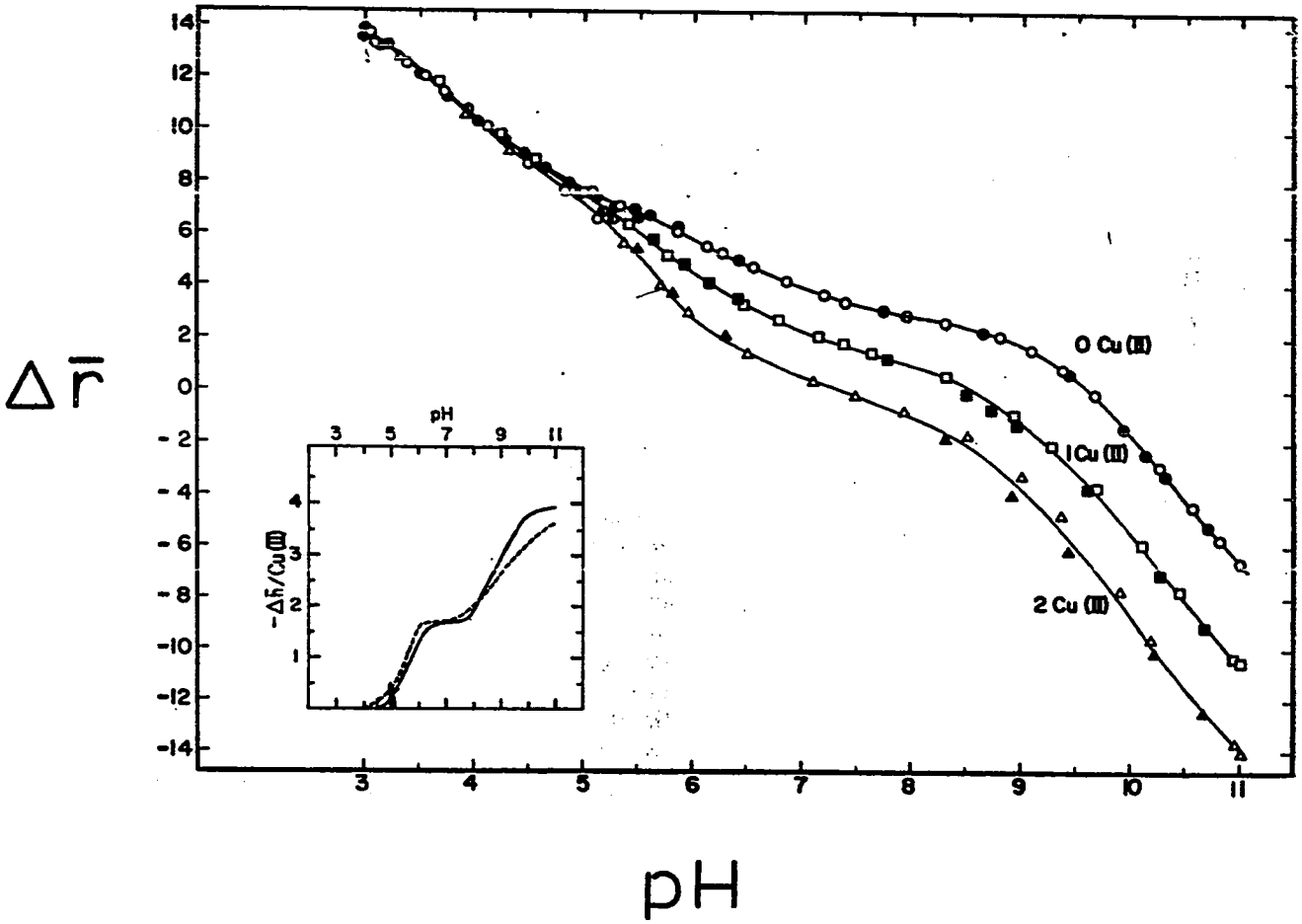

Δr̄
pH
0 Cu (II)
1 Cu (II)
2 Cu (II)
-Δr̄/Cu(II)
pH

FIGURE 5

Proton displacement from RNase by 1 equivalent of Zn(II).

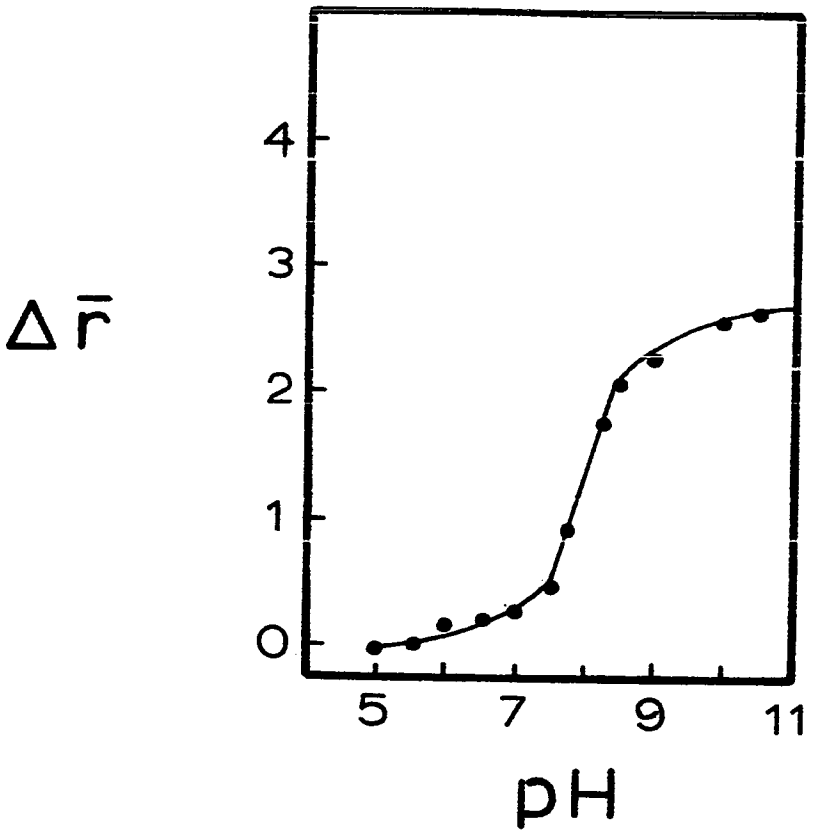

Δ r̄
4
3
2
1
0
5
7
9
11
pH

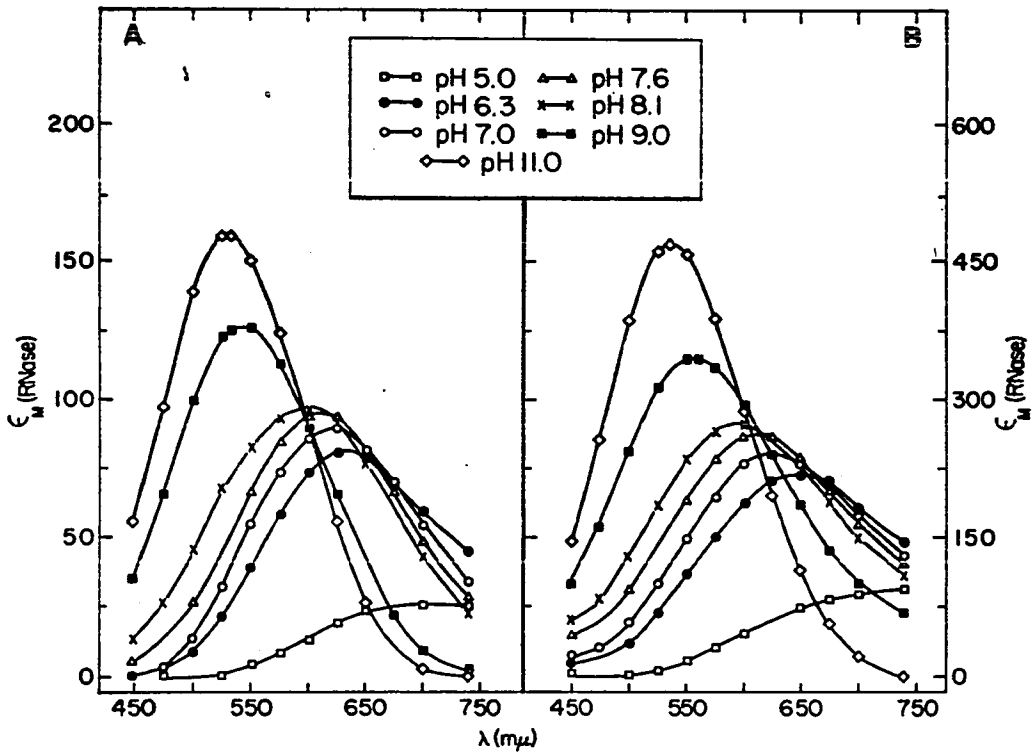
FIGURE 6

Spectra of RNase in the presence of $CuCl_2$. The conditions were:

Protein concentration of 3 to 4%; ionic strength, 0.16.

A, molar ratio of $CuCl_2$ to RNase of 1. B, molar ratio of $CuCl_2$ to RNase of 3. All data are corrected for a small amount of scattering estimated from the optical density at 450 mμ. Results are reported as the extinction per mole of RNase. The ordinate in B is 3 times the ordinate in A.

The data are from Breslow and Girotti (1966).


A
B
pH 5.0
pH 6.3
pH 7.0
pH 7.6
pH 8.1
pH 9.0
pH 11.0
200
150
100
50
0
600
450
300
150
0
450
550
650
750
λ (mμ)
ϵ_M (Ribose)
ϵ_M (Ribose)
x100

reasoning is the theoretical justification for the difference curve not returning to zero as would be the case if metal ion were bound by a single titratable ligand type with a pK value somewhere near the center of the pH scale. Identification of the ligand type with the higher pK_{int} as peptide nitrogen was made with the aid of the visible absorption spectra shown in Figure 6. Girotti and Breslow (1966) provide a table, with references, of the most probable chelate sites giving the value of λ max expected for each site. Figures 4 and 6 together provide the best fit if it is assumed that each chelate site contains one imidazole and two peptide nitrogens at the usual pH of RNase assay.

Using the mass action equations developed by Bryce et al (1965) for the binding of Cu(II) to histidyl-containing peptides and an equation similar to equation 4, Girotti and Breslow (1966) obtain a value for log K of 3.9, where K is again the overall association constant. The agreement between this value of the association constant for Cu:RNase and the value presented by Ross et al (1962) for Zn:RNase is probably fortuitous even though the agreement may mean that both constants have similar orders of magnitude. In later work where association constants for Cu:RNase were determined by Breslow (1967) using a gel filtration system, the association constants were determined for each of the four histidyl-containing binding sites

plus a fifth site. At pH 5.5, one site was found to have an affinity of 5.3×10^3 and four sites were found to have an association constant of 5.2×10^2. At pH 7.0, five sites were found to have equivalent affinities of 3.2×10^5. Saundry and Stein (1966) have also reported a pH dependent association constant for the binding of Cu(II) to RNase. These authors used equilibrium dialysis in the presence of metal ion buffers to arrive at values of K of 3.89×10^5 at pH 6.1 and 5.14×10^6 at pH 7.0. All values of the association constants discussed are apparent association constants; estimates of the true association constants necessarily depend upon assumptions concerning the nature of the chelate sites. This question will be discussed further in connection with the results of experiments presented in this thesis.

The difference titration curves in Figure 5 for the binding of Zn(II) to RNase present a more difficult problem for interpretation in spite of the fact that the curves appear to have a simple shape. Adequate model studies are not available and Breslow and Girotti (1966) favor the interpretation that the portion of the curves below pH 7 arises from an interaction different from that producing the remainder of the curve. In their view, it is not possible to interpret the titration of the additional protons above pH 7, and the simplest explanation of Figure 5 was said to be the assumption of a 1:1 complex formation between Zn(II) and imidazole groups.

D. Denaturation of RNase

The general behavior of RNase in denaturing media is well-documented. Harrington and Schellman (1956) showed that reversible denaturation occurs in 8M urea and that reversible thermal denaturation occurs over a temperature range of about 45° to 70°. If the protein is held at temperatures higher than 65° over an extended period of time, irreversible denaturation occurs (Bigelow, 1961; Hermans and Scheraga, 1961 a). Anfinsen et al (1955) found that 5 M guanidine hydrochloride plus 1.2 M urea produced changes similar to those observed in 8 M urea. Extreme pH values are also observed to denature RNase. Solutions at a pH of about 11 or higher produce irreversible denaturation of the protein; solutions at pH 2 or lower cause reversible denaturation if the temperature is held below 45°.

The denaturation of RNase in 8 M urea has produced some particularly interesting results. It is consistently observed (Sela and Anfinsen, 1957; Barnard, 1964 a, b) that the unfolding of the protein is markedly time-dependent; depending on the pH, up to 30 minutesor longer may be required for complete denaturation. This medium is then well suited for kinetic studies of the denaturation of RNase. Such studies in urea solution have been carried out by Barnard (1964 a, b). Since Anfinsen (1956) and Anfinsen et al (1955)

showed that RNase is enzymically active in 8 M urea and various workers (Harrington and Schellman, 1956; Sela et al, 1957) had demonstrated that the protein alone is extensively unfolded in this medium, it was believed that the substrate was capable of refolding the protein molecule into its active configuration. Barnard (1964 a, b) was able to show, however, that the process of refolding by substrate is due to preferential binding of phosphate by the molecules in the active configuration rather than direct interaction between phosphate and unfolded protein. Substrate analogues, including phosphate ion, are also able to counteract the denaturation of RNase in 8 M urea. Barnard (1964 a, b) measured the effects of many of these analogues and his treatment of the data is similar to one of the analyses to be presented here of RNase destabilization by Cu(II).

The existence, referred to above, of three anomalous tyrosyl residues has greatly facilitated the study of the denaturation of RNase. Such anomalous tyrosyl residues are not unique to RNase but the existence of a total of only six tyrosyls, plus the absence of tryptophyl residues, has made the interpretation of ultraviolet spectral denaturation data for RNase much easier, than for proteins containing mixtures of these chromophores.

Using the ultraviolet difference spectrum technique, Bigelow and Geschwind (1960) have measured the effects of some

common protein denaturants, as well as the effects of some simple salts, on the spectrum of tyrosine. Similar work has been done by Wetlaufer, Edsall and Hollingworth (1958) and by Donovan, Laskowski and Scheraga (1958), in which the pH of solutions of chromophoric amino acids was varied. In all this work, it was shown that the composition of the medium produced definite shifts in the spectra of the amino acids. Ultraviolet difference spectra have also been observed for RNase in denaturing media by a number of workers, notably by Sheraga (1957), Sela and Anfinsen (1957), Bigelow and Geschwind (1960), and by Bigelow (1960, 1961). Since difference spectra are observed for the protein, it follows that the environment, or effective medium, in which the tyrosyl residues are found changes during protein denaturation. Bigelow and Geschwind (1960) then see the process of denaturation as one in which the three anomalous tyrosyl residues are transferred, by protein unfolding, from a position within the protein fabric to one in contact with the solvent. This idea is supported by the general proposals of Williams and Foster (1959), who consider most denaturing media to be of a lower refractive index than the protein interior. A blue shift, or shift of the absorption maximum to a shorter wavelength, is expected if a tyrosyl residue is transferred to a medium of lower refractive index. Such a blue shift is observed during the denaturation of RNase

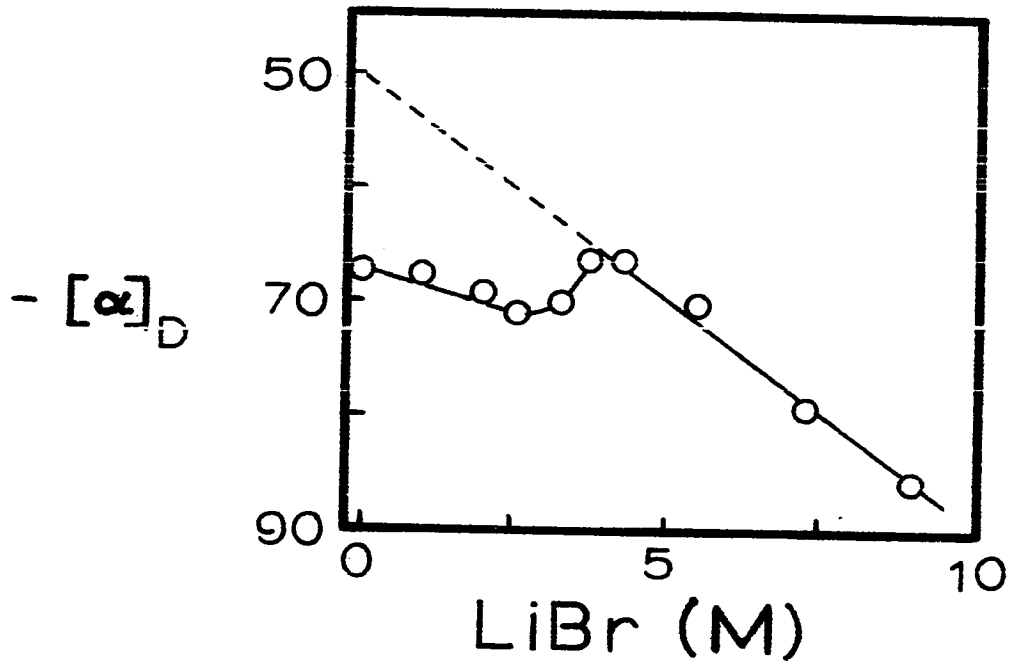
and other proteins, and is now commonly referred to as the "denaturation blue shift". It is significant that Herskovits and Laskowski (1960), using their solvent-perturbation technique, found three solvent-accessible and three non-accessible tyrosyl residues in native RNase.

It may then be expected that, if one places samples of native RNase, or any other protein, in solutions of varying concentrations of denaturant, there will be two sources of contribution to the shape of a plot of total change, $\Delta\varepsilon$, in molar absorptivity vs. denaturant concentration. One contribution will be from the denaturation blue shift; the other will be from a continuous shift of the tyrosyl spectrum caused by the variation in composition of the medium. These shifts are referred to as solvent effects by Bigelow (1960) and may be to shorter or longer wavelengths, depending on the nature of the denaturant, i.e., urea, pH, temperature, etc. If the effect is linear with concentration of denaturant, as is frequently observed (Bigelow, 1960, 1964), extrapolation of the portions of the denaturation curve on either side of the denaturation blue shift to zero concentration of the denaturant allows an accurate estimate of the total value of the denaturation blue shift. Such an estimate is not otherwise possible. Figure 7 (Bigelow, 1964) shows one example of such an extrapolation of data from the denaturation of RNase

FIGURE 7

Specific rotation of ribonuclease as a function of LiBr molarity.

From Bigelow (1964).


- [α]D
50
70
90
0
5
10
LiBr (M)

by solutions of LiBr. In this particular instance, optical rotation was used to measure the extent of denaturation. The data chosen for Figure 7 point up the general utility of the extrapolation procedure. Linearity in solvent effects has been observed not only when measurements were made by ultraviolet difference spectra or optical rotation, but also in the case of viscosity measurements (Bigelow, 1964). However, linearity is not always observed (Sarfare and Bigelow, 1967).

Some further definition of the term "solvent effect" is desirable. Since, e.g., consideration of the refractive index of the denaturing medium is not pertinent when viscosity measurements are being taken, the term may be used in a quite general sense. It must also be emphasized that even when optical measurements are taken, the change in refractive index of the effective medium is not the only possible origin of what has been termed solvent effects, e.g., Brandts(1964), considering thermal denaturation of chymotrypsinogen, sees the heat capacity of the polymer as a function of temperature. Such changes in the heat capacity could be of importance in contributing to the difference absorption in the wavelength regions on either side of the denaturation blue shift and could affect the slope of the extrapolation lines to the starting temperature.

Since most workers define denaturation as the production

of any molecular conformation which is grossly different from the single conformation defined as the native state, it would be surprising if different denatured conformations, or states, could not be produced for a given protein in different denaturing media. This does not necessarily mean that protein in any given denaturing medium passes through more than one discrete conformation with an energy minimum; the denaturation equilibrium is typically viewed as involving only the native and denatured conformations (Schellman, 1955; Brandts, 1964 a, b). There are many exceptions however. Hermans and Scheraga (1961 a) chose to interpret RNase thermal denaturation as occurring in two steps, each step possessing its own unique thermodynamic parameters. Riddiford (1966) offers good evidence that the denaturation of paramyosin occurs as the sequential formation of different denatured states. In this case, the steps are clearly distinguishable in plots of extent of denaturation vs. concentration of denaturant.

In the case of RNase, the extrapolation procedures greatly facilitate the identification of the denaturation state that the protein assumes in a given medium. Bigelow (1960) showed that each of the three anomalous tyrosyl residues contributes a definite change to the total change in molar absorptivity at 287 mμ upon protein denaturation. The denaturation state attained by RNase may then be assigned as

states I, II, or III depending upon whether tyrosyl B, B and A, or B, A and C are normalized. The value of the denaturation blue shift for tyrosyls B, A, and C are about 700, 1,000 and 1,000 molar absorptivity units respectively. It was shown (Bigelow, 1964) that denaturation states I, II and III may also be identified by virtue of their possessing definite values in viscosity and optical rotation changes. Bigelow (1964) has published a compilation of the results of various workers from which he identified the denaturation state attained by RNase in various media. This table is reproduced here as Table 1.

It is not assumed that there can be only three possible denaturation states for RNase. There could be more than three, but the existence of additional states has not been detected. It is possible that, if Hermans and Scheraga (1961 a) are correct in their interpretation of the thermal denaturation process, a fourth denaturation state exists which does not give rise to gross changes in the viscosity, optical rotation, or ultraviolet absorption of the protein. Alternatively, detectable amounts of denatured state I might be formed in equilibrium with native RNase before the production of state II begins.

Of particular interest are studies of destabilization effects by materials which are able to lower the concentration of denaturant

TABLE I

DENATURED CONFORMATIONS OF RIBONUCLEASE AND THEIR PROPERTIES

State	Denaturant	$\Delta\epsilon_{287}$*	$[\eta]$ (dl./g)	$\Delta[\eta]$** (%)
Native	---	0	0.033	0
I	pH 1 at 15°C	-700 (B)	0.035	7
II	pH 6 at 60°C	-1700 (B and A)	0.065	21-23
	pH 1 at 40°C	-1700 (B and A)	0.072	24
	5 M-LiBr	-1700 (B and A)	0.073	23
	pH 2, μ=0.1, 2 M-dioxane	-1700 (B and A)	0.070	23
III	8 M-urea	-2700 (B, A and C)	0.093-0.095	46
	6 M-guanidinium chloride	-2700 (B, A and C)	0.094	51

* $\Delta\epsilon$ 287 is the value of the change in molar extinction at 287 mμ corrected for solvent effects. The letters B, A and C identify the tyrosyl residues which contribute to it (Bigelow, 1960, 1961).

** $\Delta[\eta]$ is the percentage increase in the specific rotation of the denatured form compared to the native form.

needed to complete the transition from the native to the denatured form. Depending on the amounts needed of such materials, the destabilization can be classified roughly as a specific or a medium effect. When very low molar ratios of destabilizer to protein are required, it can be assumed that destabilization occurs as a result of the binding of the destabilizer by protein. Some examples of materials which have a destabilizing effect on RNase are various alcohols (Schrier, Ingwall and Scheraga, 1965; von Hippel and Wong, 1965), and many common electrolytes (von Hippel and Wong, 1965). The stabilizing effect of phosphate and sulfate has already been noted and is well characterized by Ginsburg and Carroll (1965).

III. MATERIALS AND METHODS

A. Materials

1. Ribonuclease

Two commercial samples of RNase were used throughout this work. The first of these was obtained from Sigma Chemical Co., Lot 768-8511, and was used for all experiments except those involving the kinetics of formation of RNase derivatives. Protein for the latter experiments was a product of Mann Research Laboratories as Lot S1684. Both companies claimed their product to be homogeneous RNase A produced by the method of Crestfield et al (1963 a) with the only difference in preparation being that the Sigma sample was lyophilized from acetic acid solution whereas the Mann sample was the ethanol precipitate.

Checks on the purity of both samples were carried out as described in a later section.

2. 1-carboxymethylhistidine-119-ribonuclease

The RNase derivative was purchased from Mann Research Laboratories as Lot L2739.

3. Cupric Nitrate

Cupric nitrate was from Baker and Adams reagent grade lot. The material was stated to be of better than 99.5% purity and was used as received. Stock solutions of Cu(II) were made at 5.15 x 10^{-2}M in 0.1 M KCl at pH 2.0.

4. Zinc Nitrate

This reagent was of an unknown source and purity and was therefore standardized using the E.D.T.A. titration method outlined by Fischer (1961). Stock solutions of Zn(II) were prepared at 7.84 x 10^{-2}M in 0.1 M KCl at pH 2.0. Analysis of these solutions showed that the starting material was better than 99% pure.

5. Potassium Hydroxide

Mallinkrodt Chemical Works reagent grade KOH was dissolved in water to make a solution of about 5 M. A carbonate-free solution for titration purposes was obtained by the method of Powell and Hiller (1957). In this procedure, the concentrated KOH solution is treated with a sufficient amount of $Ba(OH)_2$ to precipitate all of the carbonate as $BaCO_3$. The solution is then filtered and passed

through a 4.0 x 30 cm column of Amberlite IR-120 in the potassium form. The effluent KOH solution is free of carbonate and chloride ions and may be further diluted for titration purposes.

All steps in this procedure were carried out under nitrogen. Final solutions of KOH for titration were adjusted so that they were 0.16 M in KCl. KOH solutions were stored under nitrogen in a polyethylene bottle fitted with an air inlet tube containing ascarite.

6. Urea

Urea solutions were prepared by dissolving the appropriate amount of Mann Ultra Pure Urea, Lot R-3280, in water. The solutions were slightly turbid and were passed through a 0.45μ Millipore filter in a Swinnex 25 filter holder mounted on a syringe. Solutions so treated were clear and showed less absorbance at 287 mμ than did solutions of 3x recrystallized urea prepared in this laboratory by the method of Levy and Margoliash (1962).

Millipore filters and Swinnex filter holders were obtained from the Millipore Corporation.

7. Iodoacetate

Two sources of iodoacetate were used in this work. Sodium iodoacetate was a product of Sigma Chemical Co. and required purification to remove free iodine, as it had been in storage

for well over a year. A suspension was made in reagent grade acetone and stirred for 15 minutes at 25°. The bulk of the iodine was taken up in the acetone and could be removed by filtration of the suspension through Whatman No. 1 filter paper on a Buchner funnel. The nearly white powder was resuspended in acetone, and cautious additions of water with intermittent stirring were made to dissolve the powder. The solution was placed in the freezer to allow crystallization to take place. Suction filtration gave white plates. The product was dried by prolonged suction, followed by a 24 hour drying period at 40°.

Iodoacetic acid was a product of Eastman Distillation Products Industries. This material was nearly iodine-free as evidenced by its color and was used as received.

Preliminary experiments on the carboxymethylation of RNase were carried out using the Sigma product; experiments reported in this thesis were performed using the Eastman product.

8. Other Reagents

Reagents not specifically covered above were reagent or analytical grade and were used as received.

B. Methods

1. Purification of Ribonuclease

The extent of purification of the RNase samples used in this work depended partially on the experiment for which the protein was intended. However, since both the Mann and Sigma lots gave solutions which were turbid, filtration was always necessary as a first step. In the early stages of the work, RNase was dissolved in 0.1 M KCl to give about 3% by weight concentration. These solutions were then passed over a column of G-75 Sephadex which had been poured from a suspension of G-75 in 0.1 M KCl. If care was taken in the application of the sample to the column, about 90% of the protein eluted at a concentration of 0.2% or greater. Various sizes of columns were used as the amount of protein desired at a certain concentration varied with the experiment, but the elution conditions were always chosen to correspond to an elution rate of 30 ml/hr on a column 2.4 x 35 cm.

An alternative method of filtration was used during the later stages of this work. In this procedure, protein solution was made up in the concentration desired and filtered through a 0.45μ Millipore filter in a Swinnex 25 filter holder mounted on a 10 ml syringe.

In addition to providing non-turbid protein solutions for optical measurements, the use of the Sephadex column also gives information about the state of aggregation of the protein in solution. It is known that RNase aggregates may be detected as a fast moving peak on Sephadex G-75 and that these aggregates may be thermally reversed, if they have arisen from lyophilization of acetic acid solutions (Crestfield et al, 1962). As shown in Figure 8, determined using the Sephadex G-75 column referred to above, the Sigma preparation as initially received contained either very little or nearly 100% aggregate. Figure 9 shows the elution pattern, on the same column but with a flow rate slowed to 7 ml/hr, of an equimolar mixture of the Mann and Sigma preparations after the Sigma lot only had been heated to 65° for a 10 minute period. This mixture was chromatographed at the close of the research and indicates that the maximum amount of thermally reversible aggregate must have been negligible in either commercial sample at any time throughout this research. The 0.1 M KCl medium may have been effective in the elimination of aggregate (Crestfield et al, 1962).

Although the Sephadex chromatography does give useful information, a better check on the purity of any given sample of RNase is provided by ion-exchange chromatography on BioRex 70 using the methods developed by Hirs, Moore and Stein (1953) and by

FIGURE 8

Gel Filtration of Sigma RNase A on Sephadex G-25. 100 mg protein was applied and eluted in 0.1 M KCl at 30 mls/hr on a column 2.4 x 35 cm.

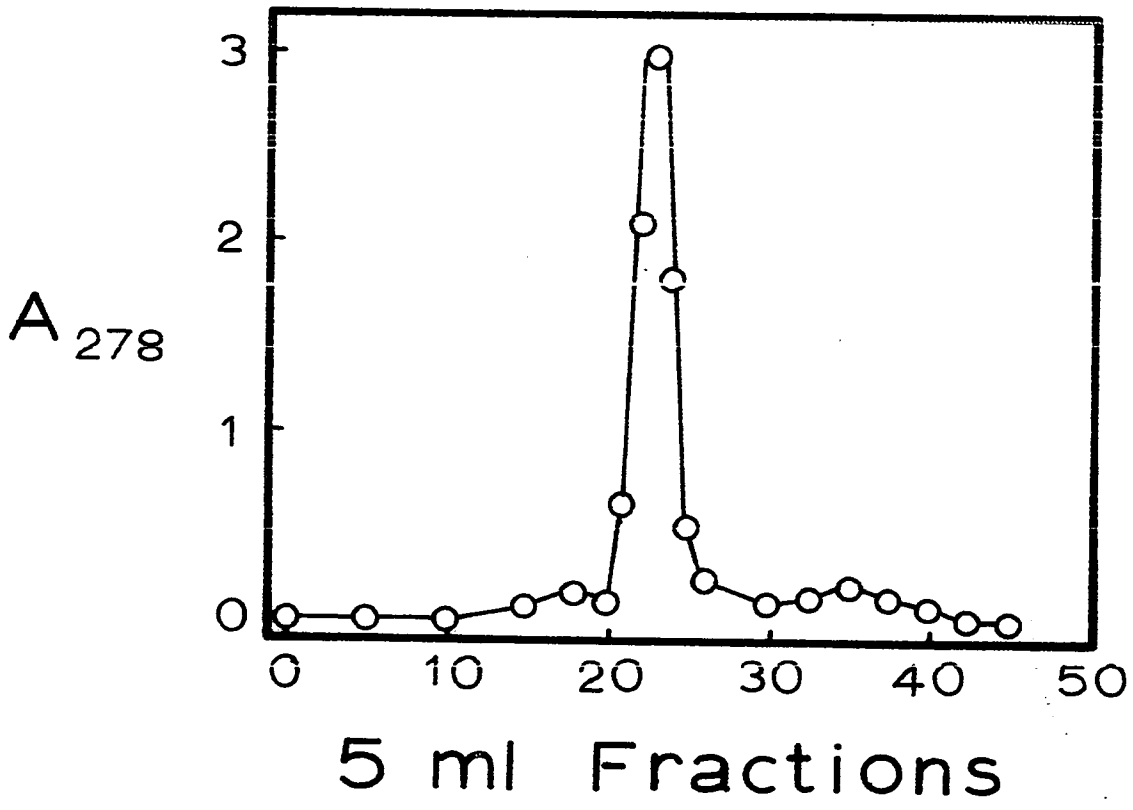
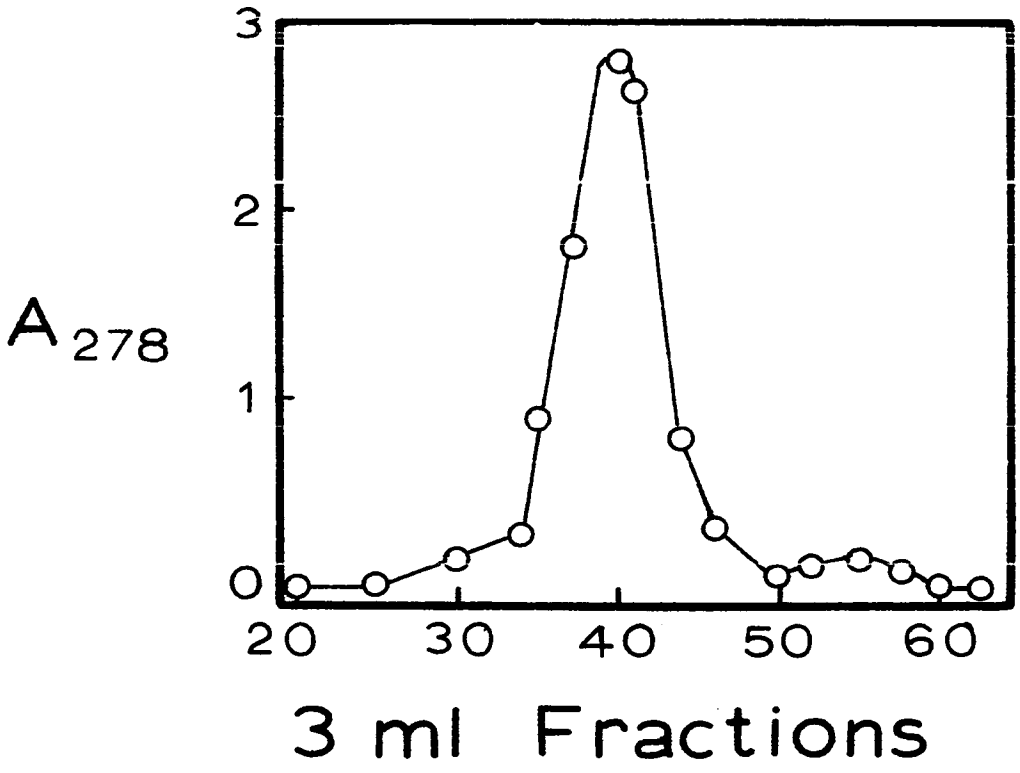

A 278
3
2
1
0
0
10
20
30
40
50
5 ml Fractions

FIGURE 9

G-25 Filtration of an equimolar mixture of Mann and Sigma RNase A. 100 mg protein was applied and eluted at a flow rate of 7 mls/hr in 0.1 M KCl. E.A. A'zary carried out the experiment.


A 278
3
2
1
0
20
30
40
50
60
3 ml Fractions

Crestfield, Stein and Moore (1963 a). Both the Sigma and the Mann lots of RNase were chromatographed by one of these methods prior to their use. Figure 10 shows the elution pattern of the Sigma RNase as initially received. Figure 11 shows the condition of the protein after about 9 months storage in the dry frozen state. It can be seen that significant changes have occurred in the homogeneity of the protein.

The Mann preparation was analyzed on BioRex 70 immediately before its use and all experiments with it were finished within a four-week period following this chromatographic purity check. Figure 12 shows the state of homogeneity of this preparation after a 10 month storage in the dry frozen state. The Mann lot was apparently less subject to deterioration.

Both the Sigma and Mann preparation were thermally deaggregated prior to ion-exchange chromatography and both gave quantitative yields within experimental error. Thus both were apparently free of any appreciable amount of aggregate, since neither the reversible nor the irreversible aggregate is elutable from BioRex 70.

It was also important to know whether significant amounts of divalent anion, principally phosphate ion, were bound to the histidyl residues of the two RNase preparations. For this reason,

FIGURE 10

BioRex 70 chromatography of Sigma RNase A. 50 mg protein was applied to a 2 x 50 cm column and eluted at a flow rate of 30 ml/hour in 0.2 M phosphate buffer at pH 6.45.

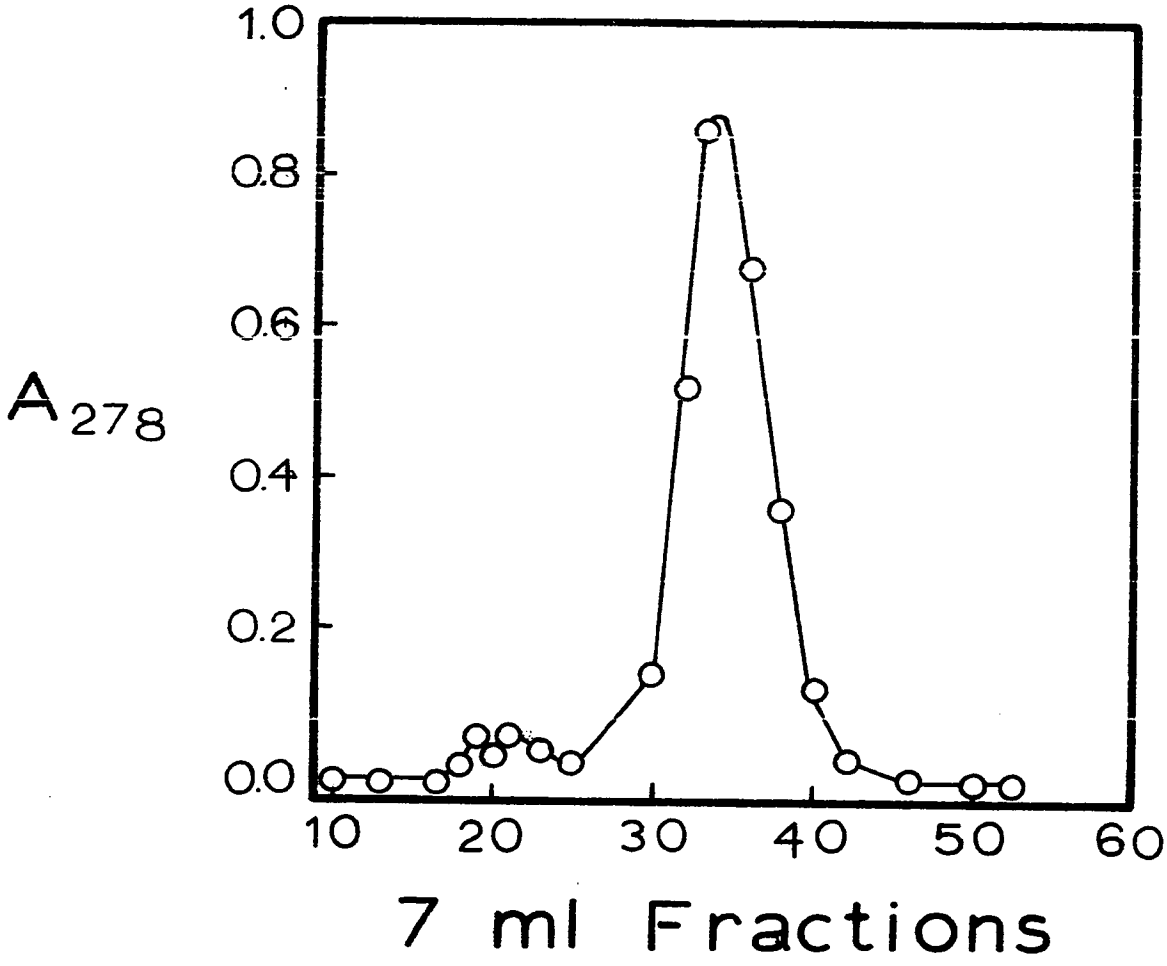

1.0
0.8
0.6
0.4
0.2
0.0
A278
10
20
30
40
50
60
7 ml Fractions

FIGURE 11

BioRex 70 chromatography of Sigma RNase A after 9 months storage. 10 mg protein was applied to a 1/2 in x 35 cm and eluted at 26 ml/hr in 0.2 M phosphate buffer at pH 6.47.

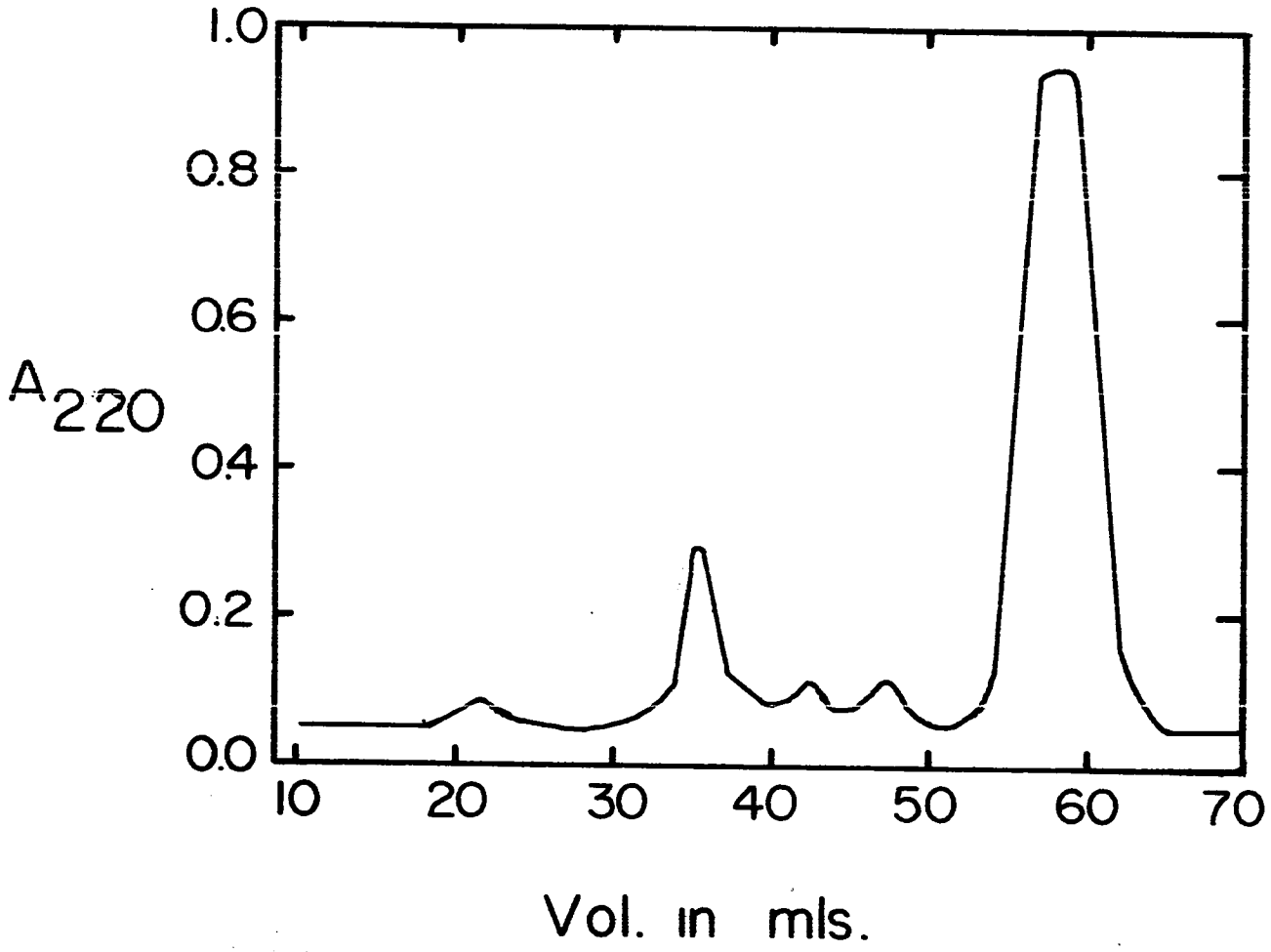
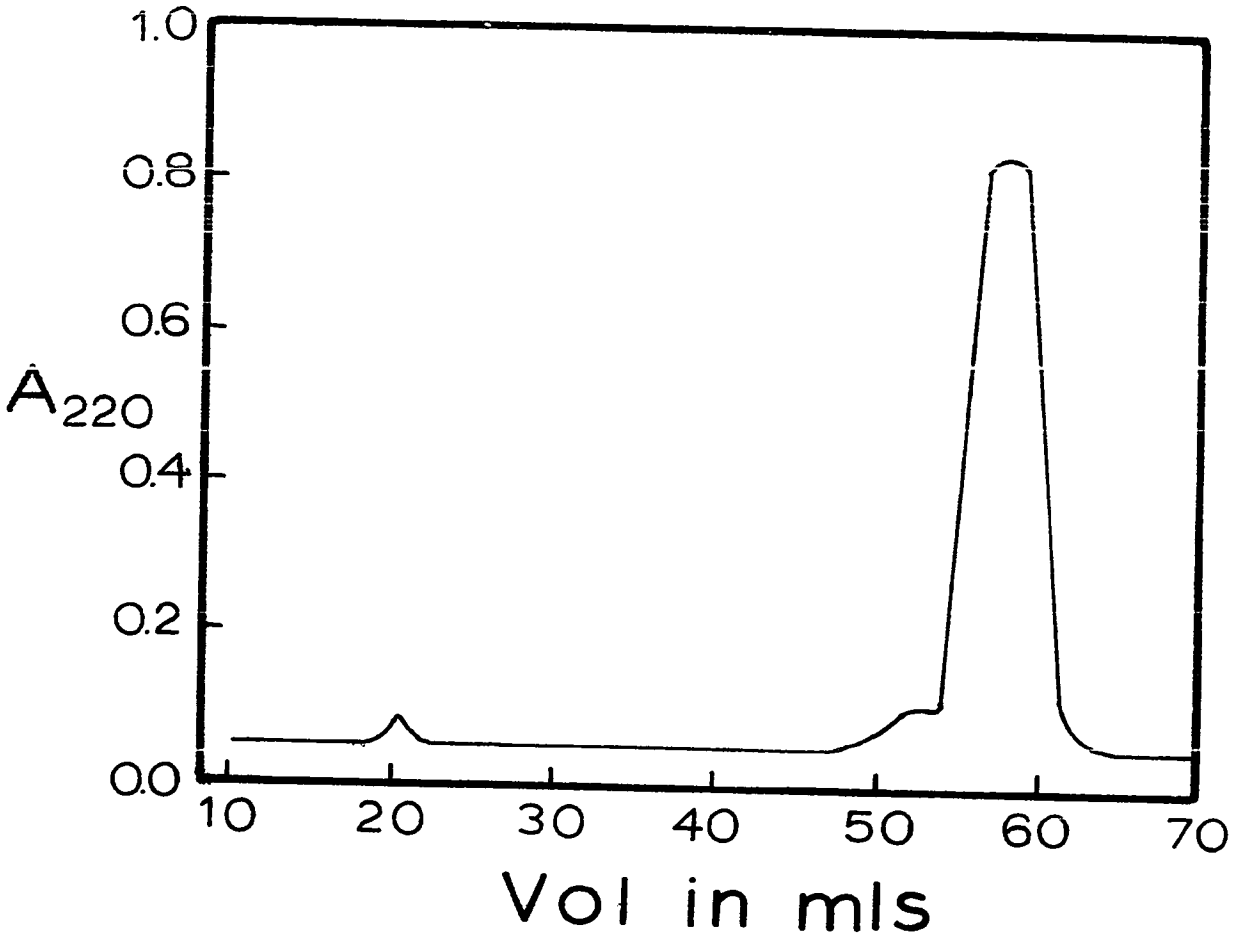

1.0
0.8
0.6
0.4
0.2
0.0
A_{220}
10
20
30
40
50
60
70
Vol. in mls.

FIGURE 12

BioRex 70 chromatography of Mann RNase A. Conditions were the same as for the chromatography of Sigma RNase A (Figure 11).


1.0
0.8
0.6
A220
0.4
0.2
0.0
10
20
30
40
50
60
70
Vol in mls

titration curves were produced for the Sigma lot of RNase A. Figure 13 shows the results of the titration, compared to the titration curve for RNase published by Tanford and Hauenstein (1956). It can be seen that little, if any, divalent ion was bound to the protein. The estimated error in $\bar{r}$ in the region of histidyl titration is ± 0.25 at the titration conditions employed. It may also be seen that the sample appears normal throughout the titration curve.

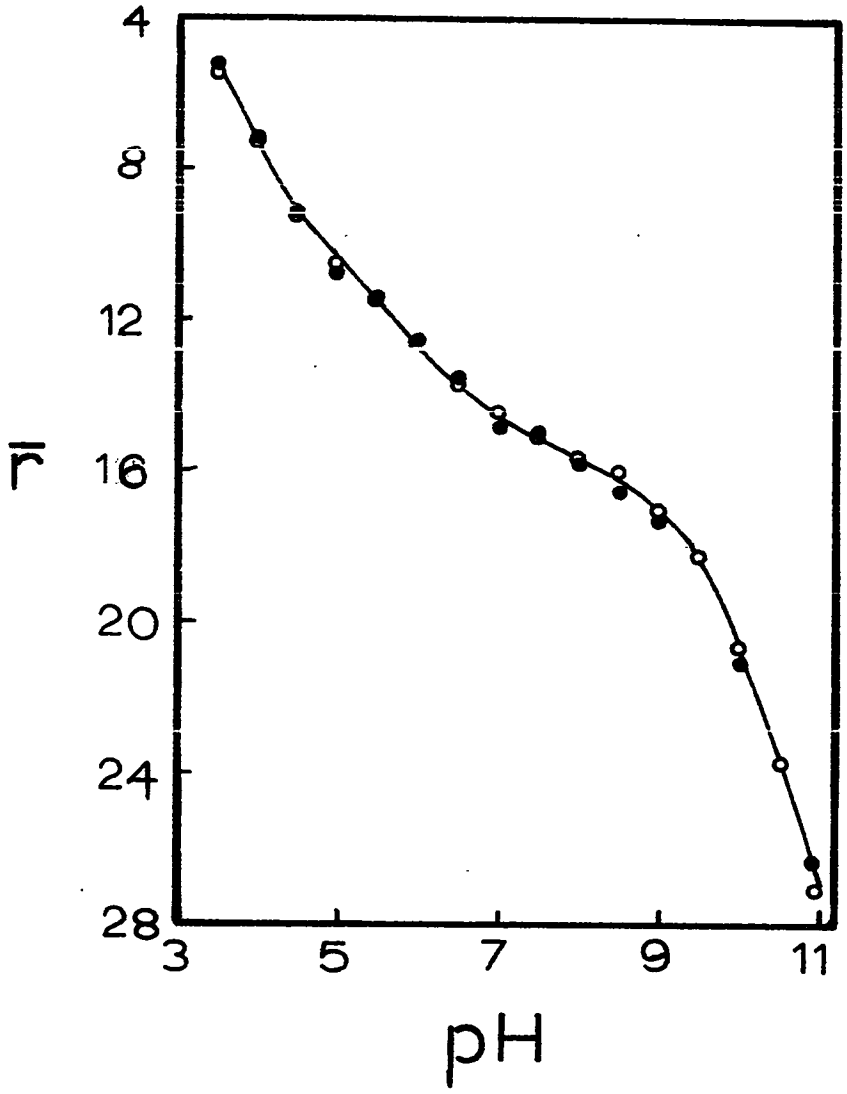
It was not considered necessary to carry out the titration for the Mann sample of RNase since this lot of protein was only used in the production of RNase derivatives. Since derivatives were formed at rates comparable to values published for RNase A free of phosphate and sulfate ions, it was assumed that the Mann lot of RNase A was also free of such ions.

2. Storage of Ribonuclease

Except where noted otherwise, all RNase samples for various experiments reported in this thesis were stored in 0.1 M KCl. If any given solution was to be used within a 2-3 week period, it was stored at 4°. Protein which could not be used within this time period was frozen in solution until use. It was found that insoluble, irreversibly formed, aggregate was present in solutions which were kept longer than 2 months in the frozen state. Such protein was neither analyzed nor used in experiments.

FIGURE 13

Continuous titration of Sigma RNase A in 0.16 M KNO_3. 2.5 ml of a 1% solution of protein were titrated with 0.384 N KOH. The open circles are experimental points; the closed circles are data from Tanford and Hauenstein (1956).


4
8
12
16
20
24
28
r̄
3
5
7
9
11
pH

Since the Sigma preparation of RNase A was shown to be subject to some degree of deterioration, certain key denaturation experiments were repeated using the Mann preparation. Appendix I summarizes the results of these experiments and shows that no significant differences are obtained between the two lots. Thus the products of deterioration of the Sigma RNase A are not detectably different in their denaturation or metal-binding behavior.

3. Ion-Exchange Chromatography

With the exception of the results shown in Figure 10, all the ion-exchange chromatography experiments on BioRex 70 were carried out using the rapid elution scheme of Crestfield, et al (1963a). Figure 10 was obtained from 2 x 50 cm column of BioRex 70, 200 mesh. Fifty mg of the Sigma lot protein, by dry weight, was manually applied in a 5 ml volume and eluted at a flow rate of 30 ml/hr using 0.2 M phosphate buffer at pH 6.45. The effluent was monitored by its absorbance at 278 mμ, using a Beckman DU spectrophotometer.

Rapid elution experiments were carried out on a 1/2 in x 35 cm column of BioRex 70, minus 400 mesh. Fines were eliminated from the resin as outlined by Crestfield, et al (1963a). Material which did not sediment in phosphate buffer at a rate greater

than 0.2 cm/min was discarded. The resin was sized and equilibrated in a beaker by successive additions of 0.2 M phosphate buffer at pH 6.49. Prewashing with solutions of HCl and NaOH was omitted.

The column used was a product of Chromatronix Inc., model no. LC-1/2-43, and was equipped with a Chromatronix SV-8031 sample injection valve. The column was packed from a slurry of BioRex 70 in 0.2 M phosphate buffer, pH 6.49, of about 60% buffer content by volume. Due to the compression of the resin bed when the fine mesh resin was used, it was necessary to condition the column under pressures higher than those used during the actual experiments. Even under this greater packing pressure, several additions of slurry were required, and the column length was repeatedly adjusted using the bed supports supplied with the column.

Conditioning and elution pressures were obtained with the use of a Milton-Roy Mini-Pump, model CH 1-B-86R. The pump was adjusted to a flow rate of 35 ml/hr for conditioning and 20 ml/hr for elution. Sample loads varied between 5 and 15 mg protein and their elution patterns were obtained using a Beckman DB Spectrophotometer equipped with a Beckman linear recorder. The spectrophotometer was set to record at a wavelength of 220 mμ and

contained a Beckman 97290 Micro-Aperture Flowcell in the sample cell compartment and a Beckman 104186 Variable Beam Attenuator in the reference cell compartment.

Microorganism growth on the BioRex 70 column was controlled by passing buffer containing 0.05% sodium azide through the column between chromatographic runs. The azide absorbs strongly at the wavelength used for chromatogram recording and was eluted from the column prior to a day's experiments.

Buffer supplied to the column via the Mini-pump was deaerated under vacuum following passage through a 0.45 μ Millipore filter mounted in a Millipore XXIO 047 00 pyrex filter holder.

Although the BioRex 70 system described above is basically that of Crestfield, et al (1963 a), the flow rate used, 20 ml/hr, is only 67% of that used by these authors. Figure 29 shows chromatograms obtained using the slower flow rate. If these are compared with the chromatograms of Crestfield, et al (1963a), where all other conditions are comparable, it can be seen that the slower flow rate allows a marked increase in resolution.

4. Titration Curves

When Cu(II) binding to RNase was being measured, pH-stat measurement of protons released by metal ion was performed. When Zn(II) ions were used, continuous titration curves were recorded.

Both types of measurements were made with the aid of the Radiometer TTTI-b automatic titrator, equipped with an SBR-2c recorder, SBU-1a syringe buret, and type TTA31 micro-titration assembly. The metal ground shield on the micro-titration assembly was modified to permit its retention in the assembly with a jacketed titration cell. It was not necessary to add salt to the circulating coolant as recommended by Radiometer in lieu of the presence of the ground shield. As a substitute for the reagent reservoir and delivery system supplied by Radiometer, a 4 in length of 1/8 in O.D. Tygon tubing connected a Radiometer type 04331 capillary delivery tip directly to the syringe buret. Temperature control was maintained by means of a Haake circulating pump cooled by tap water. All solutions in the titration cell were kept under a stream of nitrogen from a cylinder of purified nitrogen.

In a typical continuous titration, 2.5 ml of protein solution of concentration between 0.7% and 1% was placed in the titration cell. The pH of this solution was adjusted to about 3.0 and the desired amount of Cu(II) added in less than 250 μl additional volume of solution. the pH of the solution was then readjusted to about 2.0, and the end point was set to this pH on the titrigraph. The instrument was then set in motion to record the titration curve. To record a measure of the reversibility of titration, the 0.384 N KOH solution

in the syringe buret was replaced by 0.411 N HCl solution and the instrument was adjusted for titration reversal. Similar titrations were performed on solutions containing all species except metal ion and protein. These curves provided a measure of the blank. After both the blank and sample curves are corrected for the continuous volume change in the titration cell, a titration curve can be calculated as the moles of H^+ released or bound vs pH. All solutions involved in titration were 0.16 M in KNO_3.

When the radiometer assembly was used as a pH-stat the volumes and concentrations of all materials remained the same with the exception of the titrant, which was diluted by a factor of 10. The titration cell contained all materials except the metal ion. The pH of the solution corresponded to the end point set on the instrument. Thus additions of metal ion caused the pH-stat to add sufficient KOH solution to restore the initial pH of the protein solution. If measurements are taken at a sufficient number of pH values, a titration curve may be constructed from the data. However, in this instance, the curves represent only the difference in protons released or bound in the presence of metal ion as compared to the value in the absence of metal ion.

Although both of the methods described here should give identical curves, it was found that under the conditions employed,

the pH-stat method tended to give higher precision. The pH-stat method was then used for all experiments involving Cu(II) ion, except when checks on reversibility of titration curves were desired. For this purpose, continuous titration was used.

The concentration of RNase for titration experiments, as well as for all other work described in this thesis, was determined by light absorption at 277.5 mμ, using the Cary 15 spectrophotometer.

5. Thermal Denaturation

Thermal denaturation was measured by ultraviolet difference spectroscopy using the Cary 15 spectrophotometer equipped with factory-designed fittings to allow heating of the cell in the sample compartment. High-temperature cells used were a product of Quaracell Products, Inc. and consisted of standard 1 cm path-length rectangular cells with a permanently cemented block-top which accepted a thermometer.

To record the progress of thermal denaturation, solutions of protein of identical pH and concentration were placed in the sample and reference cells and heated according to a definite schedule. The reference cell was a conventional 1 cm rectangular cell covered with a plastic top. The concentration of RNase was routinely between 2.0 and 2.5×10^{-4} M in 0.1 M KCl medium. This concentration allowed

for 10 minutes and then rapidly cooled. It was convenient to use the Radiometer micro-titration assembly attached to a Haake circulating pump for this purpose. The water in the Haake pump was cooled with water at 4°; the flow of coolant to the Haake pump was discontinued during the heating step. Protein solutions subjected to this rapid heating cycle were then thermally denatured in the manner outlined above, and were identical in composition to solutions whose thermal denaturation behavior had already been observed spectrophotometrically.

The addition of Cu(II) to the protein solutions was made by one of two methods. The difference consisted basically of the presence or absence of metal ion in the reference solution. When metal ion was to be present in the reference solution, sufficient solution containing the desired amount of protein and metal ion in 0.1 M KCl was prepared and the pH was adjusted to the desired value. Both cells were filled with this solution and thermal denaturation was carried out. If Cu(II) was not to be present in the reference solution, two solutions were prepared of identical composition except that a volume of 0.1 M KCl was added to the reference solution in an amount equal to the volume of metal ion solution added to the protein solution which is placed in the sample cell. Thus the concentration of protein remained identical in both cells.

the largest value of the difference in absorbance between native and denatured protein that could be measured on the Cary 15 as a negative pen deflection with the denatured protein in the sample cell compartment.

The heating schedule used involved a temperature increment each 15 minute period. It was found that the cell-heating arrangement provided with the instrument was relatively inefficient and such heating periods were necessary to ensure reproducible results. Apparently, uneven heating occurred and the 15 minute period was required to attain near-equilibrium throughout the cell. Since the lack of precision using the shorter time periods was a random effect, time-dependent denaturation was not being observed. Or, at the most, a slight amount of time-dependency could have been involved.

Two different methods were used to check the reversibility of thermal denaturation in the presence of metal ion. The first of these was simply to allow any given sample to cool following a routine measurement of thermal denaturation. The difference spectrum was again recorded after the solution had cooled to 30^{o} or less and the result compared with the initial difference spectrum at that temperature. The second method involved a rapid heating of a solution of RNase to 65^{o}. The solution was held at that temperature

The data obtained in these experiments were calculated by a procedure to be outlined in a later section.

6. Urea Denaturation

(a) Measurement by Ultraviolet Difference Spectroscopy

Ultraviolet measurements were taken as the difference in absorption using the Zeiss PMQII spectrophotometer. This instrument was not equipped for temperature control and all measurements were taken at ambient temperature using 1 cm rectangular cells. Solutions for difference measurement were prepared in the manner described above at a protein concentration of about 2.0×10^{-4} M. Both the sample and reference cells contained protein and Cu(II) in 0.1 M KCl, at identical concentrations. The sample cell only contained urea at the concentration desired for the particular measurement. All solutions were adjusted to pH 5.5, and a 45 minute time period was allowed before measurement so that the time-dependent denaturation was complete.

Measurements of the absorption difference were taken at 287 mμ and at a series of additional wavelengths at values between 300 and 400 mμ. Correction was made for the slight absorbance of the urea. The determination of the absorptivity difference due to the denaturation blue shift was carried out in the same manner as will be outlined for thermal denaturation data.

(b) Measurements by Spectropolarimetry

For these experiments, a Jasco Model OR-UV 5 recording spectropolarimeter was used with a 50 mm pathlength cell. Solutions containing RNase A at a concentration of 1.25 mg/ml, urea at 3.15 M, KCl at 0.1 M, and metal ion at a range of concentrations were adjusted to pH 5.55 and placed in the polarimeter cell. Temperature control was not available on the instrument itself, but all solutions were kept at 25° and pH 5.55 in the thermostated titration assembly vessel described previously for a period of 45 minutes prior to measurement. This allowed for completion of time-dependent denaturation in urea solution as well as for the attainment of thermal equilibrium. Transfer of the equilibrated solutions and measurement of the optical rotation at 436mn were carried out as rapidly as possible.

7. Kinetics of Formation of Carboxymethylated Ribonucleases

Three different experimental methods were used to measure the kinetics of formation of carboxymethylated RNase derivatives. Two of these procedures allowed the separation of the products of the carboxymethylation reaction while the third gave a measure of the overall kinetic rates.

The overall rates were followed by measuring the rate of proton release during the reaction with the aid of the pH-stat. In these experiments, 3 mls of a 1.88×10^{-3} M solution of RNase in

0.1 M KCl were placed in the micro-titration assembly. To this solution, appropriate amounts of Cu(II) were added and the pH was adjusted to either 5.5 or 7.0. Iodoacetate, 0.5 ml of a 1.07 M solution, was then added; this solution had been previously adjusted to either pH 5.5 or 7.0 and was also prepared in 0.1 M KCl. The pH-stat was turned on as the iodoacetic acid was added and proton release as a function of time was recorded over a 45 minute period for each reaction measured. The temperature of the reaction solution was maintained at 30° by the Haake circulating pump.

At the end of the 45 minute reaction period, each of the reaction solutions for which kinetic determinations had been obtained were analyzed electrophoretically on Gelman Sepraphore III strips using a Gelman No. 51170 electrophoresis chamber obtained from the Gelman Instrument Co., Ann Arbor, Michigan. The buffer was Gelman's High Resolution Buffer diluted to 2200 mls. The sample was applied to Sepraphore III strips by means of a Gelman 51220 sample applicator; one application was made to each of six strips for each reaction solution. The six strips were positioned in the electrophoresis chamber and developed at 400 volts, 9 mamps, for 1 hour. The strips were then stained with a 0.1% solution of napthol blue black. Excess stain was removed by 4 rinses with 5% glacial acetic acid in 1:1 water:methanol. The strip was then dehydrated by a methanol rinse, cleared with 10% glacial acetic acid in methanol for

45 seconds and oven-dried for 15 minutes at 60°. Quantitation of the bands on the electropherograms was achieved with the use of a Photovolt Model 525 transmission densitometer, equipped with a Model 542 Photovolt Densicord.

The BioRex 70 ion-exchange system of Crestfield, et al (1963 a) offers a way of comparing the kinetics observed in this work with those obtained by other workers, since this method of product analysis has been used by various workers (Crestfield, et al, 1963 b, c; Heinrikson, Crestfield and Moore, 1965; Heinrikson, 1966). When product analysis by ion-exchange was to be carried out, 2.5 mls of solution 1% in RNase, 0.6% in iodoacetate were adjusted to pH 5.5 and allowed to react at 25° over a period of hours. No Cu(II) was present in the reaction solution and no KCl was present. The reaction mixture was placed in the micro-titration assembly and constant temperature was maintained with the aid of the Haake circulating pump. At various time intervals, samples were withdrawn from the reaction solution and inhibited by a 2:1 dilution with the 0.2 M phosphate buffer used to elute the ion-exchange column. At convenient times, a 250 µl aliquot from one of the samples was applied to the BioRex 70 column and elution carried out as described in the previous section.

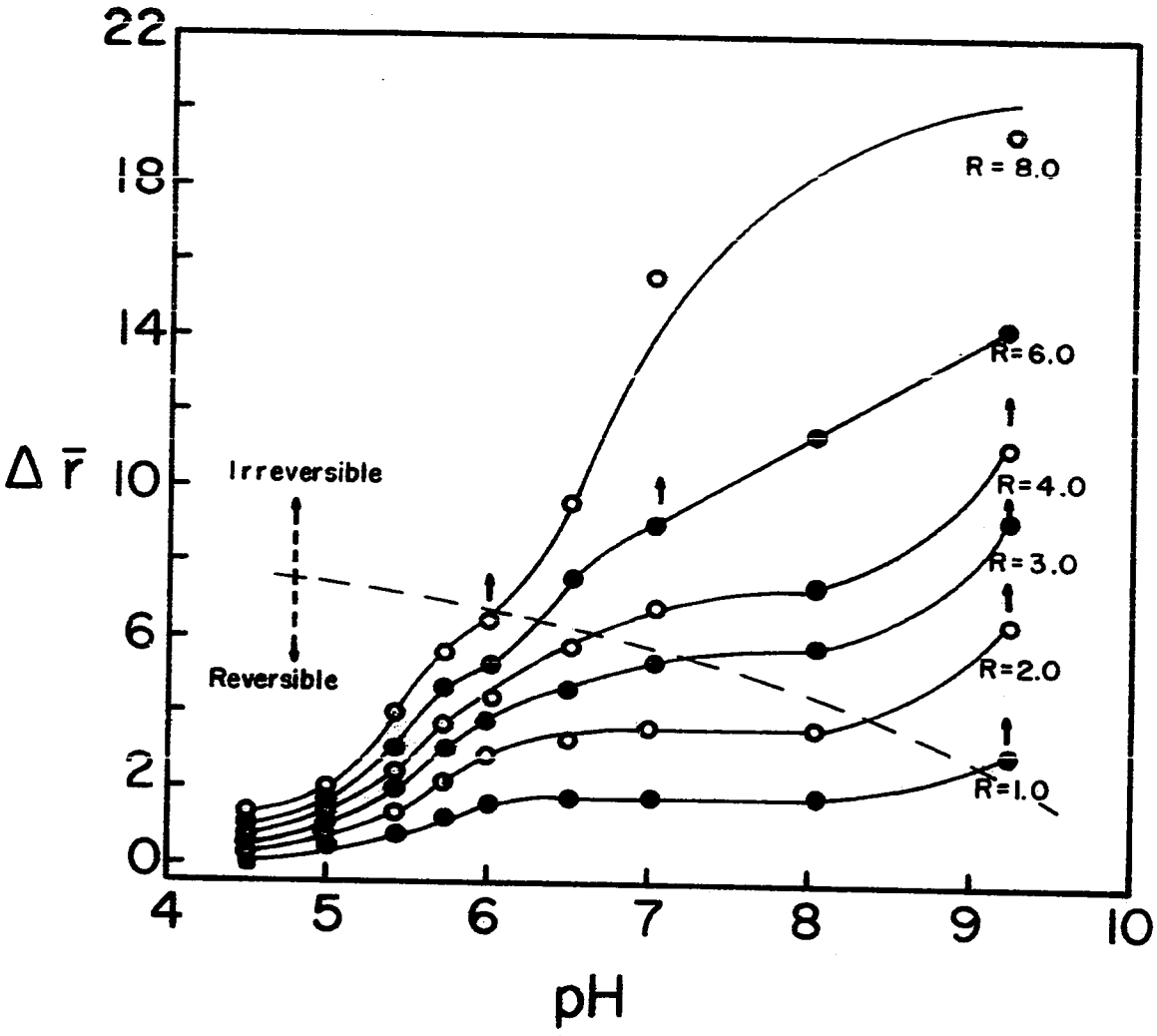
IV. RESULTS

A. Titration Curves

The difference titration curves constructed from pH-stat experiments are shown in Figure 14. In this and subsequent plots, R refers to the moles of Cu(II) per mole of RNase; i.e., R is a measure of total Cu(II). Figure 14 is divided into two areas, separated by a dotted line. This line represents the approximate limit within which RNase may be reversibly titrated in the presence of Cu(II). The area below the dotted line is that area which has been extensively investigated by Breslow and Girotti (1966) for its reversible titration behavior. It was found that if titration is carried into the area above the dotted line, complete reversibility is no longer observed. It was found in this work that titration in this area was also accompanied by a time-dependent precipitation of protein as indicated by the solid arrows in Figure 14. Breslow and Girotti (1966) have also observed this precipitation, and comment that the extent of precipitation seems to be related to the history of the protein sample. However, these authors did not observe time-dependence and, as evidenced by Figure 4, did not find values of Δr in the region

FIGURE 14

Difference titration curves for RNase in the presence of varying amounts of Cu(II). The initial concentration of RNase was 0.87%.


22
18
14
10
6
2
0
Δ r̄
Irreversible
Reversible
R = 8.0
R=6.0
R=4.0
R=3.0
R=2.0
R=1.0
4
5
6
7
8
9
10
pH

of hysteresis to be as large as those reported in Figure 14. However, if Breslow and Girotti (1966) had carried out pH-stat titration rather than continuous titration, time-dependence might have been evident in their experiments. Alternatively, such time-dependent precipitation may be exclusively due to the source and history of the RNase sample used. But, in any case, it does seem clear that additional protons are being released by Cu(II), as a result of metal binding, in the region of hysteresis. This indicates that the mode of binding of Cu(II) to RNase varies as the pH is raised. This conclusion is strengthened by the change in the visible spectrum as the pH is increased. In the region of reversible titration, the color of the solution is blue. At the onset of hysteresis the solution changes to a violet color and becomes pink as the pH is increased above 8.0. The change to the pink coloration is accompanied by increasing solubility of the precipitate.

In view of the complications presented by the region of hysteresis, it was decided that this pH range was not a fruitful one for further investigation. Therefore, the pH range of reversible binding to sites containing the histidyl residues was chosen for further study. Table 2 is a composite table of data from Breslow and Girotti (1966) and from this work; the table shows the good agreement between these two sets of data. The data covers only the area of reversible titration and is illustrated in Figures 4 and 14. It can be

TABLE 2

TITRATION OF PROTONS DISPLACED FROM RNASE BY CU(II)

R	pH 5.5		pH 7.0	
	Breslow and Girotti	This Work	Breslow and Girotti	This Work
1	.79	.8	1.7	1.8
2	1.6	1.6	3.3	3.6
3	2.4	2.2	5.0	5.3
4	2.9	2.8	6.7	6.7
6	3.7	3.6	pptation	pptation
8		4.4		pptation

seen from either of these Figures that binding of Cu(II) to histidyl-containing sites seem complete at pH 7.0 since the values of $\bar{r}$ reach a plateau at this pH. In addition, all of the first four binding sites appear to be titrimetrically equivalent. Since the first metal ion bound would not be expected to be bound exclusively at the first binding site, the data shown in Table 2 could not have been obtained unless binding is accompanied by a concurrent shift in the ionization constant for each of the first four sites. Such a continuous shift of the ionization constants could be the result of sequential or cooperative binding and it has been suggested by Breslow and Girotti (1966) that cooperative binding is involved.

B. Preliminary Thermal Denaturation Studies

The results of the titration experiments indicate that studies of the stability of the Cu(II):RNase A complex must be confined to a relatively narrow pH range. The pH of solutions examined must be high enough that sufficient metal ion is bound and yet should be such that reversibility of binding is exhibited. For this reason, most experiments were conducted either at pH 5.55 or pH 7.00. At pH 5.55, Figure 14 indicates that about half of the total metal ion added to the solution is bound. At pH 7.00, essentially all of the metal ion is bound. These conclusions are based on the shape of the

titration curves reported here and are consistent with the large magnitude of the binding constants reported by Saundry and Stein (1965), Girotti and Breslow (1966) and Breslow (1967).

There are additional difficulties presented whenever denaturation studies of protein are made in the presence of metal ion. The denaturation medium must not contain materials which are able to compete successfully with protein for the metal ion. For this reason, buffers were omitted from all solutions and denaturants were chosen which would not be expected to bind Cu(II) to any appreciable order of magnitude. For example, it will be shown that urea is not able to remove Cu(II) from RNase A to any significant extent. However, the stronger base, guanidine, was found to bind Cu(II) as an insoluble precipitate in the desired pH range and could not be used as a denaturant.

A further consideration is that if the titration curves are to be used to describe the state of binding of metal ion to protein, the denaturation medium should be as similar to the titration medium as is possible. Therefore, organic solvents are not a good choice of denaturant even though such solvents would generally not be expected to compete for metal ions.

For all of these reasons, thermal denaturation seemed to be a logical choice for stability studies of the metal:protein complex. The initial set of experiments contained metal ion in both the

sample and the reference cell. Figure 15 shows the effect of Cu(II) on difference spectra measured with the Cary 15 spectrophotometer during thermal denaturation of solutions adjusted to pH 5.5. The lower difference spectrum in the figure is a result of the usual denaturation blue shift observed in the absence of metal ion (Hermans and Scheraga, 1961 a). The two upper difference spectra were observed when Cu(II) was present in both sample and reference solutions at molar ratios, R, of 0.97 and 8.36. The difference spectra in Figure 15 were recorded near the transition temperature of the particular experiment. All the difference spectra show the expected minimum at about 287 mμ, though it is seen that other spectral effects interfere with the observation of the minimum normally observed at 280 mμ. In addition, Cu(II) causes an appreciable displacement of the entire difference spectrum to higher absorbence values. This displacement may be due partly to light scattering but is largely a result of new absorption by the Cu(II):RNase complex. Thermal denaturation is accompanied by a marked increase in the blue coloration of the solution containing the metal:protein complex. Further, aggregation alone is unlikely to cause changes in the essential features of the difference spectra (Bigelow, 1961).

If all of the data from these experiments are presented as a plot of the apparent change in molar absorptivity vs temperature, the result is as shown in Figure 16. In this figure, the molar

FIGURE 15

Effect of Cu(II) on the RNase denaturation difference spectrum. R values for the curves, from top to bottom, are: 8.36, 0.97, 0.00.

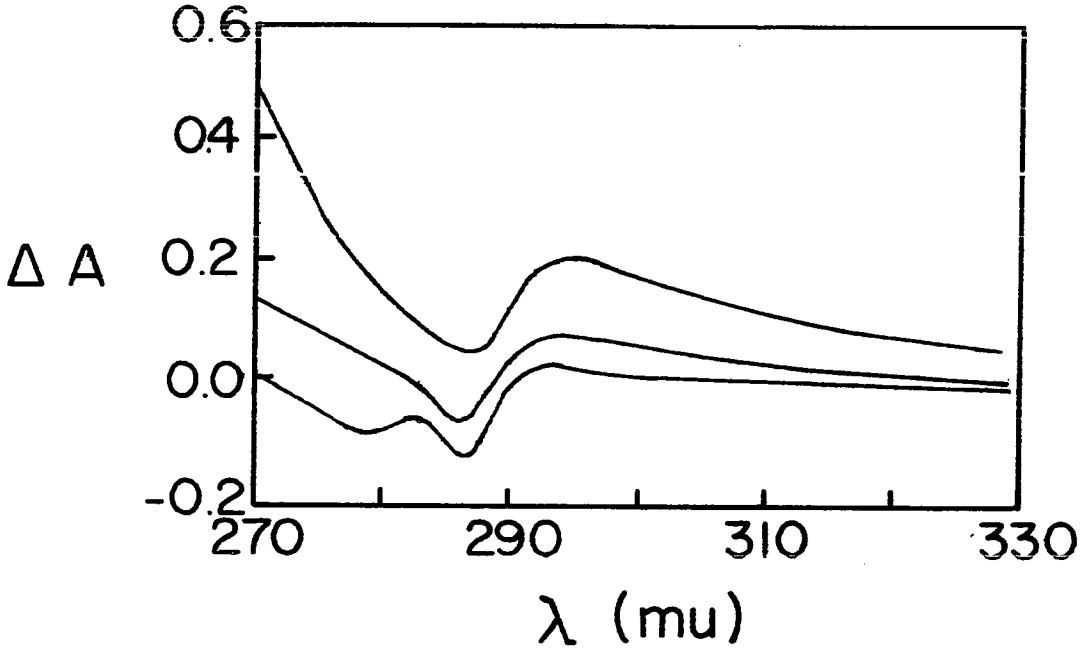
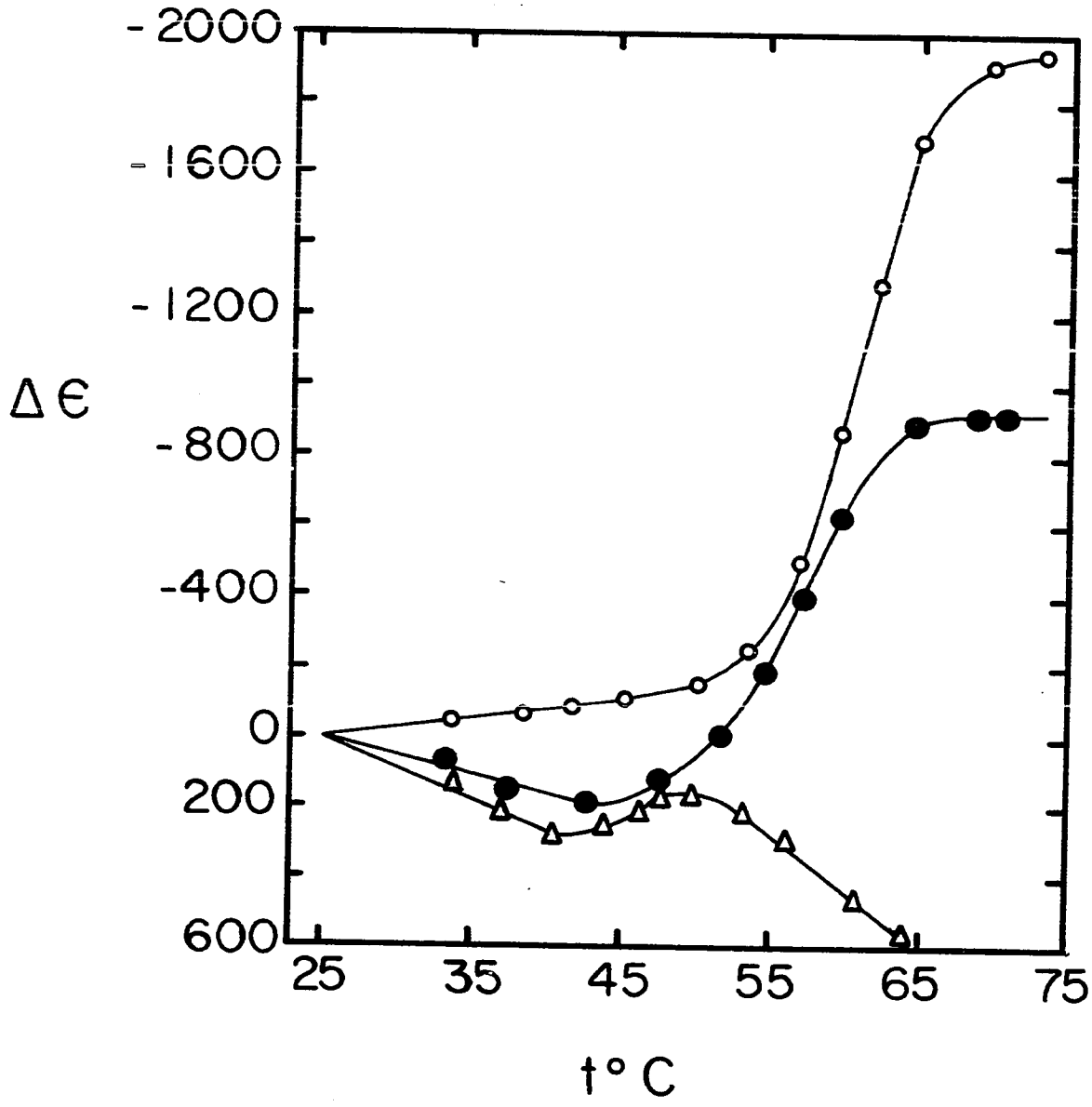

0.6
0.4
Δ A
0.2
0.0
-0.2
270
290
310
330
λ (mu)

FIGURE 16

Apparent thermal denaturation curves for RNase in the presence of Cu(II). R values for the curves, from top to bottom, are: 0.00, 0.97, 8.36.


-2000
-1600
-1200
Δε
-800
-400
0
200
600
25
35
45
55
65
75
t° C

absorptivity change, $\Delta\varepsilon$, was calculated from the change in absorbance, ΔA, relative to an initial instrument baseline of zero absorbance. ΔA, was determined at the wavelength of minimum absorbance in the region of 287 mμ. Figure 16 shows that the transition temperature, T_m, is lowered as the value of R increases. Thus the binding of Cu(II) to RNase A at pH 5.55 destabilizes the protein.

Figure 16 also appears to show that even though T_m decreases with increasing values of R, the magnitude of the denaturation blue shift also decreases as R increases. If this were true, it would contradict the finding of Bigelow (1964) that denaturation of RNase in any given medium tends to produce a certain gross denaturation state associated with a definite change in molar absorptivity. It therefore seems more likely that as the temperature is raised during thermal denaturation, the absorption of the Cu(II):RNase complex increases, resulting in a continuing displacement of the entire difference spectrum to more positive absorbance values.

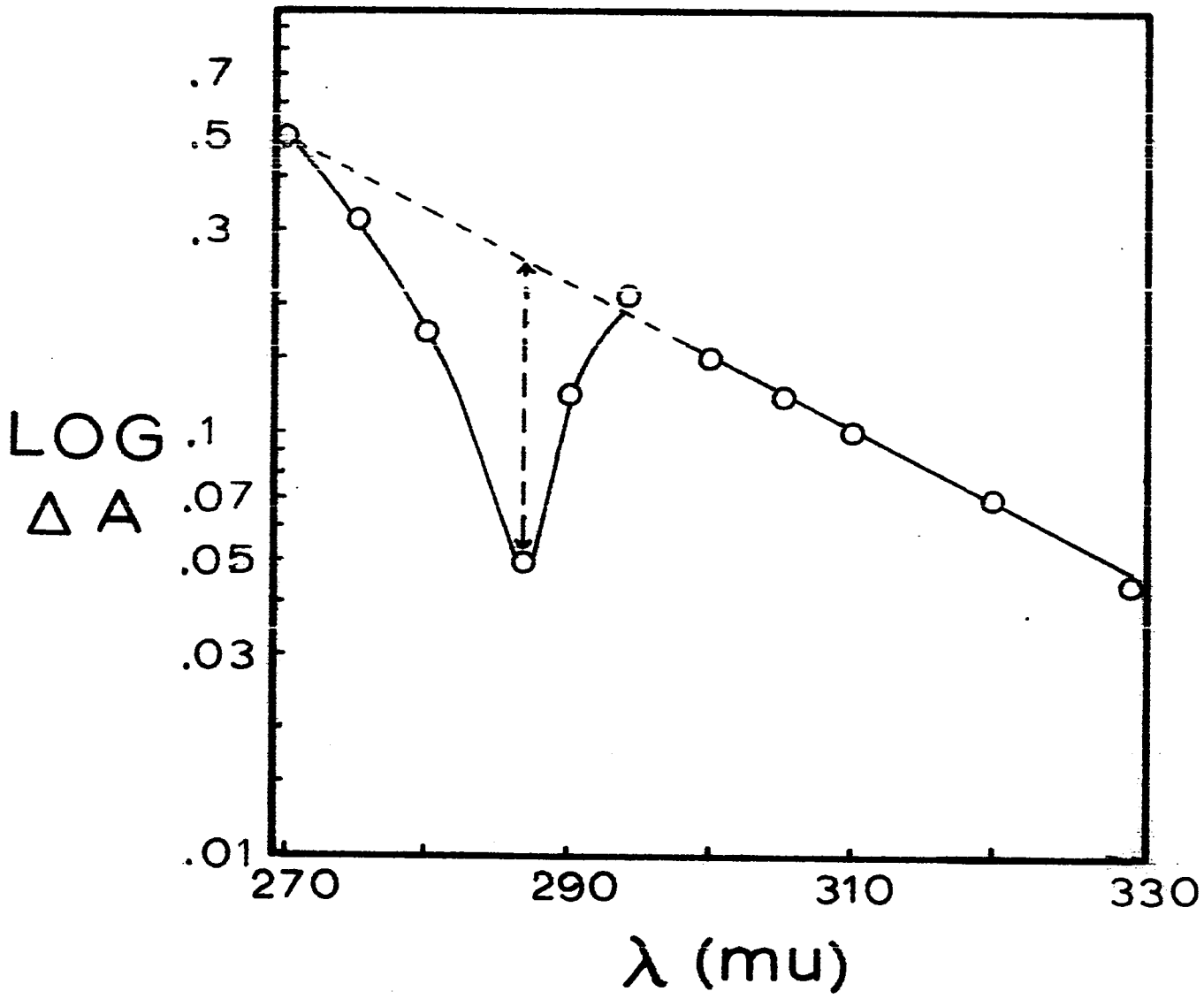
It is then necessary to find some method of subtracting the change in absorbance of the Cu(II):RNase complex from the denaturation blue shift so that the latter can be isolated. This can be done if the baseline at any temperature is defined as the difference

spectrum of the complex. The value of the denaturation blue shift is then measured relative to this new effective baseline. The only difficulty in this method is that it is not possible to determine this new baseline experimentally over the wavelength region in which the denaturation blue shift is observed. However, if some graphical procedure can be shown to rectify the difference spectrum of the complex, the absorbance value of the baseline can be determined.

It was found that a plot of log ΔA vs wavelength provided the best rectification of a number of plots tested, and tended to produce a straight line from 300 mμ to about 330 mμ. Further, this straight line tended to extrapolate directly to the absorbance value recorded at 270 mμ and provided the desired linear extrapolation across the wavelength region of the denaturation blue shift. This linear extrapolation is then defined as the baseline from which values of the denaturation blue shift can be measured. A similar type of correction has been applied to difference spectra before this (e.g., Bigelow, 1961) to remove light scattering contributions. Figure 17 shows the difference spectrum in the presence of Cu(II) at R=8.36 from Figure 15 plotted on the log ΔA vs wavelength scale. The dotted arrows in Figure 17 represent the vertical distance which corresponds to the change in absorbance resulting from the denaturation blue shift.

FIGURE 17

The linear extrapolation procedure used to determine $\Delta\varepsilon$ values for thermal denaturation in the presence of Cu(II). Data shown are for R = 8.36.

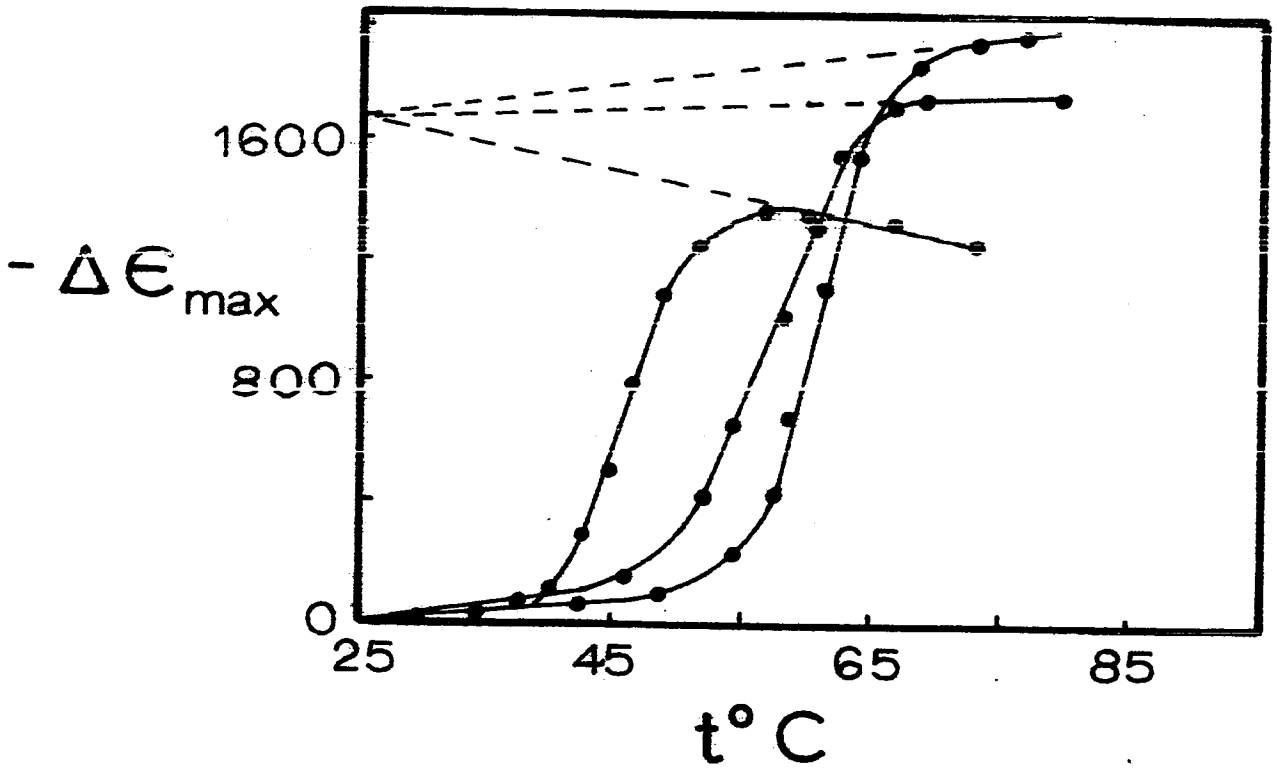

.7
.5
.3
.1
.07
.05
.03
.01
LOG
Δ A
270
290
310
330
λ (mu)

The corrected absorbance changes were converted to molar absorptivity changes and plotted against the temperature in Figure 18. The linear slope of these curves at high temperatures allows a further correction of the data for this temperature effect. If all the data are extrapolated back to 25°, they meet at $\Delta\varepsilon$ value of 1700, the value normally found for thermal denaturation (Hermans and Scheraga, 1961 a; Bigelow, 1964). These extrapolations show that the essential features of thermal denaturation are unchanged by the presence of Cu(II), and that the denatured state II, with tyrosyl residues B and A normalized (Bigelow, 1964), is still produced. The variation with R in the value and sign of the slopes of the extrapolations in Figure 17 presumably is due to changes in the spectrum of the metal:protein complex as R is increased.

It should not be inferred from Figure 17 that all of the extrapolations carried out in this work connect linearly from a point at 300 mμ to one at 270 mμ. This was true only for difference spectra recorded near and above the midpoint of the denaturation transition. Difference spectra recorded at lower temperatures show a linear segment from 300 mμ to 320 mμ on a log Δ A vs wavelength plot, but this segment does not connect with a point at any wavelength on the low wavelength side of the denaturation blue shift. This is probably not serious since there was no particular justification for designating a point at 270 mμ as one to which extrapolation should

FIGURE 18

Thermal Denaturation curves in the presence of Cu(II). R values for the curves, from left to right are: 8.36, 0.97, 0.00.


1600
800
0
$-\Delta\epsilon_{max}$
25
45
65
85
t° C

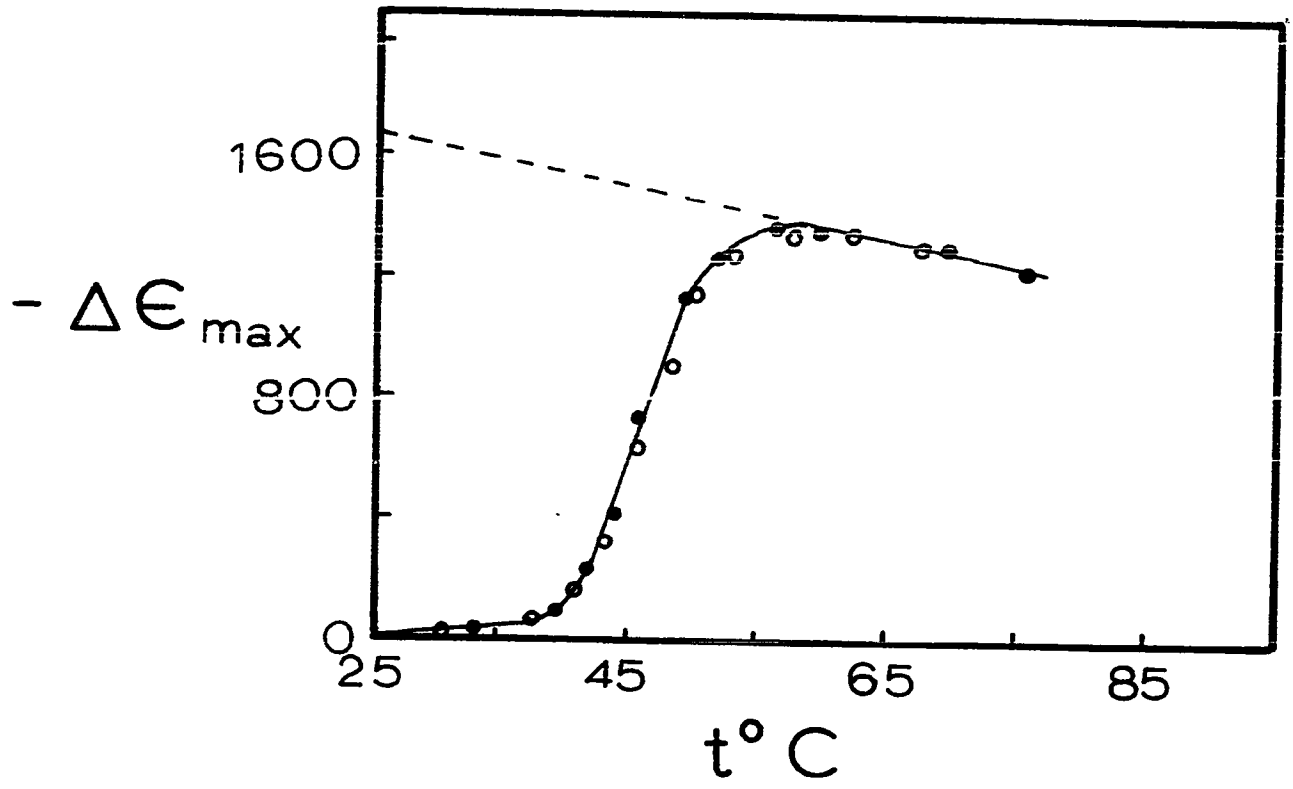
occur. Nevertheless, the low temperature range was excluded from any calculations or conclusions made from the data.

There are several reasons why the method outlined above for calculation of $\Delta\varepsilon$ values from difference spectra recorded in the presence of Cu(II) may be considered to be reliable even though the procedure is largely empirical. The most important of these is the fact that the treatment yields the expected magnitude of the total change in absorbance of RNase solutions when the protein is thermally denatured. Also, tests designed to determine unreliability of the procedure give consistently negative results. These tests are illustrated in the next section. And, finally, the results of thermodynamic calculations, to be described, are consistent with the belief that accurate values of both the transition temperature and the equilibrium constant at various temperatures were available.

Reversibility checks were carried out for the thermal denaturation experiments illustrated in Figure 18. It was found that if the RNase solution was rapidly heated to 65° and then rapidly cooled to 25°, essentially no difference was observed between its thermal denaturation behavior and that of a solution which had not been preheated. This is illustrated by Figure 19. However, after the protein had been subjected to the routine heating cycle described, only about 92% reversibility was observed. This was true over the full range of R values of 0.0 to 9.0 and undoubtedly is a result of the

FIGURE 19

Reversibility of thermal denaturation of RNase in the presence of Cu(II). Open circles refer to measurements made on a solution of RNase which had been preheated to 65° for 10 mins. and rapidly cooled. Closed circles are from an experiment in which preheating was omitted. Both solutions were identical and contained Cu(II) at R = 8.36.


1600
800
0
$-\Delta\epsilon_{max}$
25
45
65
85
t° C

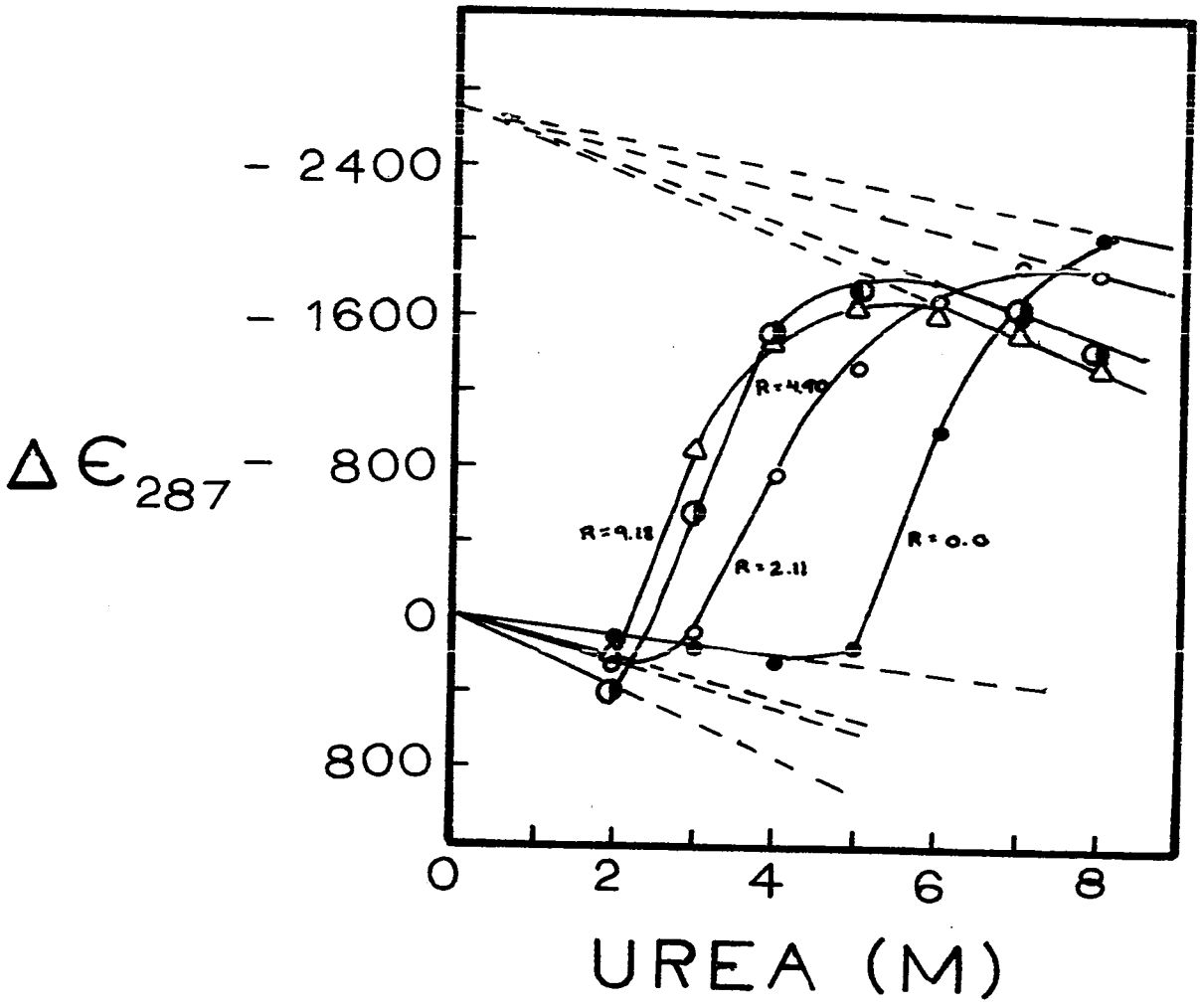
existence of the protein at temperatures above the transition temperature, T_m, for periods of an average length of 1 1/2 hours (Harrington and Schellman, 1956; Hermans and Scheraga, 1961 a). It was concluded that the presence of Cu(II) had no effect on the reversibility of thermal denaturation of RNase.

C. Urea Denaturation

It was of interest to determine whether the destabilization of RNase A to thermal denaturation by Cu(II) was unique to the method of denaturation or whether such destabilization could be observed using other denaturants. Urea was found to satisfy the criteria outlined for a suitable denaturant in the preceding section and difference spectroscopy measurements were made in a series of experiments in which both the concentration of urea and the values of R were varied. The results of these experiments are shown in Figure 20. The magnitude of the $\Delta\varepsilon$ values presented in Figure 20 were determined as outlined for thermal denaturation experiments. It is seen that again Cu(II) destabilizes the protein towards the denaturant and the metal ion can probably be said to exert a general destabilizing effect upon the RNase molecule. Again, Cu(II) is not able to produce a different denatured state than would be observed for RNase in the absence of metal ion, since urea is known to produce denatured state III with all of the three anomalous

FIGURE 20

Effect of Cu(II) on urea denaturation of RNase in 0.1 M KCl at pH 5.55.


- 2400
- 1600
- 800
0
800
$\Delta \epsilon_{287}$
R=9.18
R=4.40
R=2.11
R=0.0
0
2
4
6
8
UREA (M)

tyrosyl residues normalized (Bigelow, 1964), to produce a total change in $\Delta\varepsilon$ of 2700 units.

All of the conclusions to this point are valid so long as it is certain that no significant uncertainty is associated with the calculation of the $\Delta\varepsilon$ values. In the absence of such uncertainty, measurements of the Cu(II):RNase complex in the same denaturing medium by two different physical techniques should produce equivalent results. Therefore, a series of measurements were made with RNase in 3.12 M urea over a range of R values, using the Jasco spectropolarimeter.

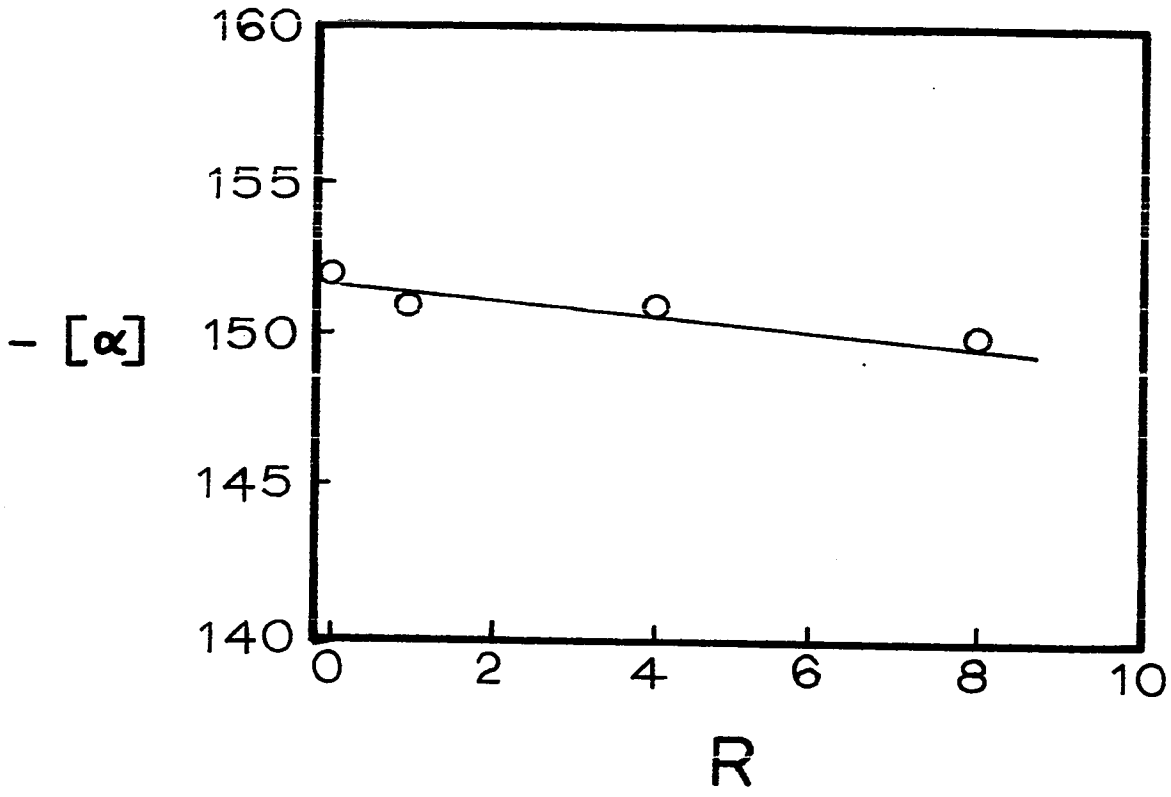
Initially, it was shown that the binding of Cu(II) does not appreciably affect the specific rotation of RNase at pH 5.5. Figure 21 shows the results of the addition of varying amounts of Cu(II) to RNase in 0.1 M KCl. Since it is also known (Foss and Schellman, 1959; Gordon and Jencks, 1963) that urea does not exert solvent effects on the rotation of RNase, it is possible to calculate values of the equilibrium constant, K, for the rotation data without recourse to measurements over a wide range of molarities of urea. K is defined as

$$K = \frac{(\text{denatured molecules})}{(\text{native molecules})} \qquad (5)$$

$$= \frac{y}{1 - y} \qquad (6)$$

FIGURE 21

The effect of Cu(II) on the specific rotation of RNase at 436 mμ.


160
155
150
145
140
- [α]
0
2
4
6
8
10
R

where y is the fraction of total change in specific rotation attained at any value of R. The total possible change in specific rotation at the completion of the transition from native to denatured RNase is taken as 46% of the specific rotation in the absence of urea (Bigelow, 1964). Similar calculations can be made from the spectroscopic data in Figure 20 except that in that case, because of the necessity of correcting the data for solvent effects, the total possible value of $\Delta\varepsilon$ is determined by measuring the vertical distance between the dotted extrapolation lines at 3.12 M urea.

Figure 22 shows the specific rotations measured in 3.12 M urea as a function of R. Figure 23 shows the results of the optical rotation and difference spectroscopy measurements plotted together as the logarithm of K vs the logarithm of R. A theoretical justification of this type of plot will be presented in a later section; its purpose in Figure 23 is to allow a linear presentation from both sets of measurements. All of the data tend to fall on the same straight line, but with a greater random spread than observed by Hermans and Scheraga (1961 a) in their demonstration of the equivalence of thermal denaturation measured by polarimetry and by difference spectroscopy.

There are three important sources of error which contribute to the random spread of the data in Figure 23. It has been shown by Barnard (1964 b) that a 2-3^{o} temperature variation around

FIGURE 22

Effect of Cu(II) on the specific rotation of RNase in 3.12 M urea and 0.1 M KCl at pH 5.55.

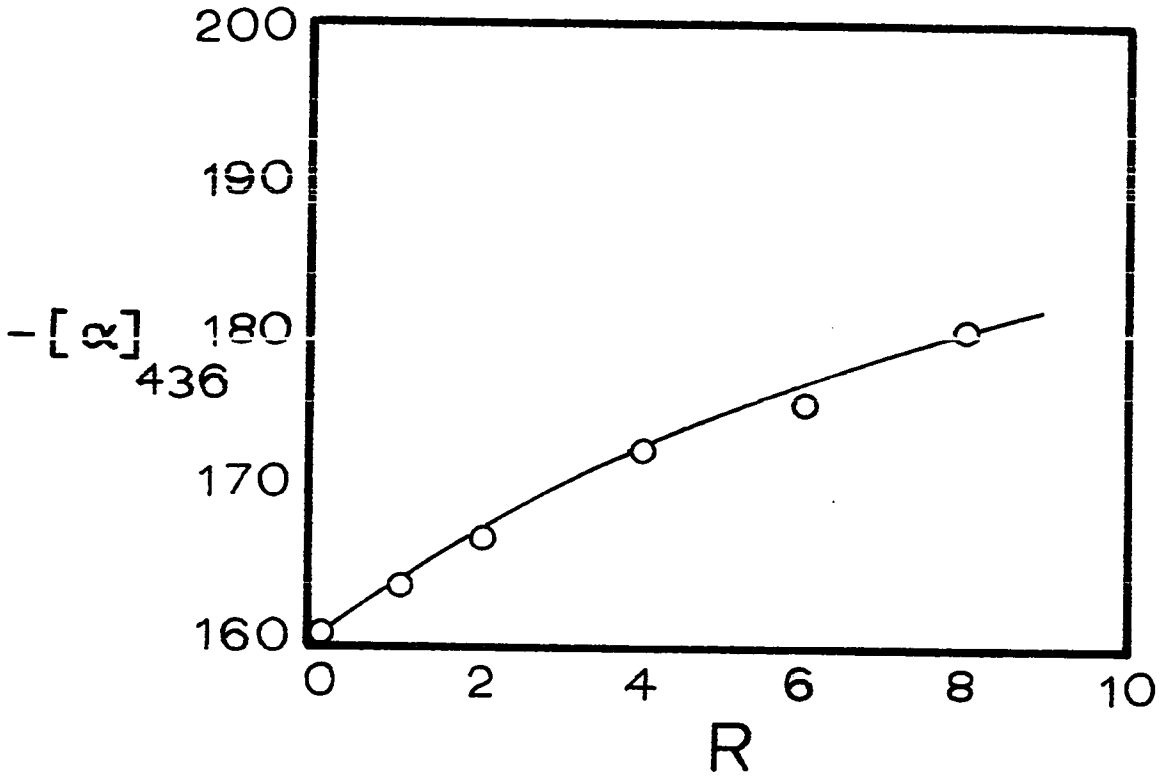
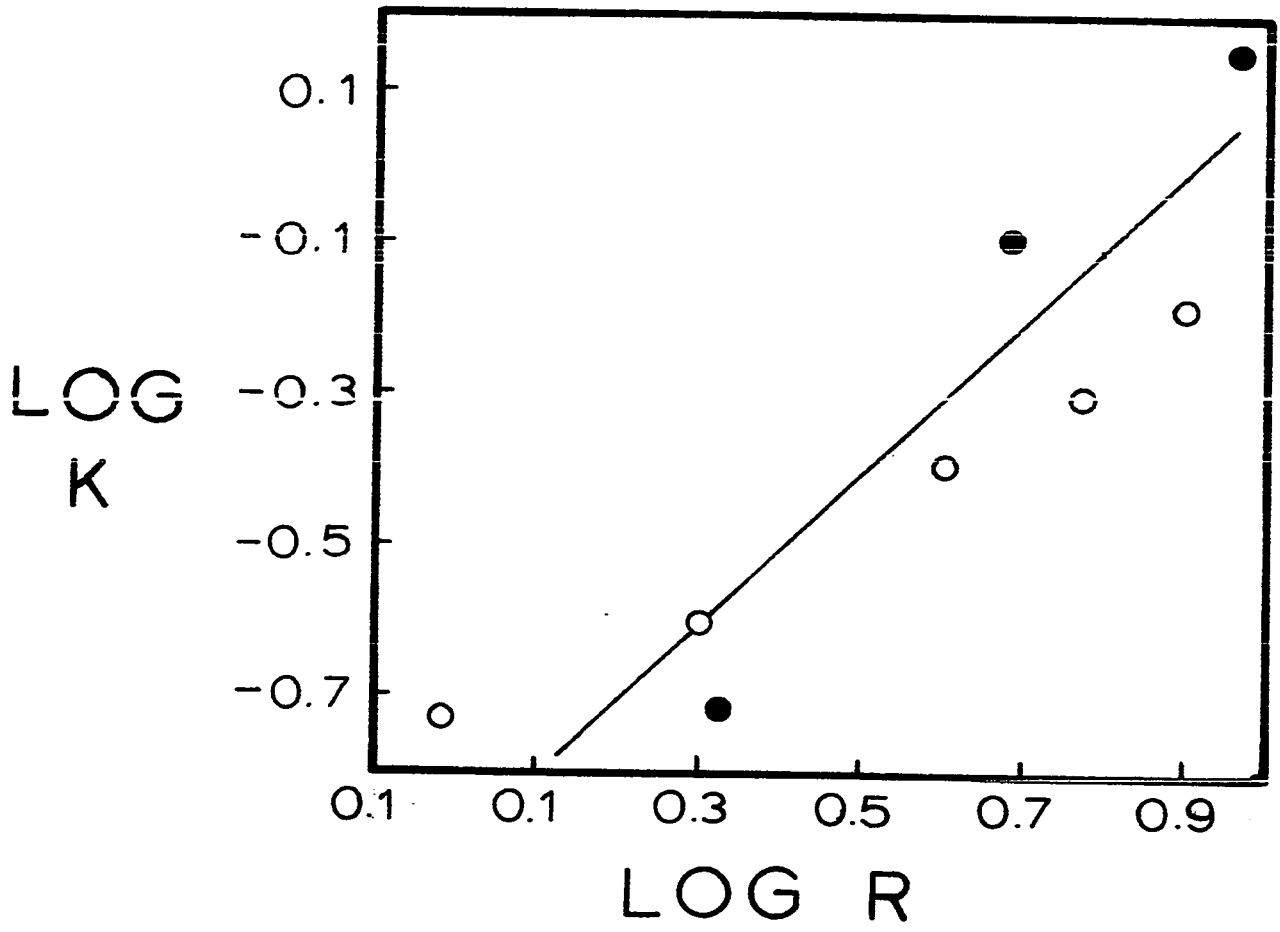

200
190
180
170
160
$-[\alpha]_{436}$
0
2
4
6
8
10
R

FIGURE 23

Comparison of optical rotation and difference spectroscopy measurements of the destabilization of RNase by Cu(II) in 3.12 M urea at pH 5.55. Closed circles are from spectroscopy measurements; open circles are from optical rotation measurements.


0.1
-0.1
-0.3
-0.5
-0.7
LOG
K
0.1
0.1
0.3
0.5
0.7
0.9
LOG R

25° can produce significant changes in the value of the equilibrium constant during urea denaturation of RNase. Since temperature control was not available on either of the instruments used in measuring data shown in Figures 20 and 22, this is probably an important source of error. Also, the assumption that the total possible change in specific rotation over denaturation transition is exactly 46% (Bigelow, 1964) is probably not entirely justified, since this value is based on only two sets of experiments. Thirdly, any errors in Figure 22 arising from inadequate temperature control will result in an additional error term when vertical measurements are made between the extrapolation lines.

It is possible, therefore, that Figure 23 may be taken as evidence of the reliability of the extrapolation procedure illustrated in Figure 17. In addition, Figures 20 and 22 may be taken as qualitative proof of destabilization. The measurements in urea were not repeated since a more significant test was devised to show that there are no apparent uncertainties contributing to the results of the thermal denaturation measurements. The results of this test are given below.

D. Thermal Denaturation Over A Range of pH Values

Since the protonation of the imidazole groups involved in the sites at which Cu(II) is bound to RNase could conceivably

affect the local environment around the binding site, it is important to determine the stability of the Cu(II):RNase complex over as wide a range of pH values as possible. In addition, the range of R values covered in Figure 17 was expanded and thermal denaturation measurements were taken with Cu(II) absent from the reference cell. If the procedure to determine $\Delta\varepsilon$ values is reliable, the same results should be observed in the absence of Cu(II) from the reference cell as are observed in its presence.

Figure 24 shows the variation in the change in the transition temperature with R at pH 5.5 when the series of experiments in Figure 18 is expanded and additional experiments performed with metal ion absent from the reference cell. It is seen that there are no significant differences in T_m values when the two types of thermal denaturation measurements are compared. Plots presented in a later section will show that there are also no significant differences in values of the equilibrium constant calculated from the two types of experiments.

Since the major interest is the stability of Cu(II):RNase relative to RNase itself, it is necessary to measure T_m values for RNase in 0.1 M KCl over the pH range considered. This was done and the results are given in Figure 25. Figure 25 is consistent with a similar curve presented by Hermans and Scheraga (1961 a) and supports the conclusion of these authors that the protonation of

FIGURE 24

Variation of T_m with R at pH 5.55 in 0.1 M KCl. The closed circles show data obtained with Cu(II) in the reference cell; open circles show data obtained when Cu(II) was absent from the reference cell.

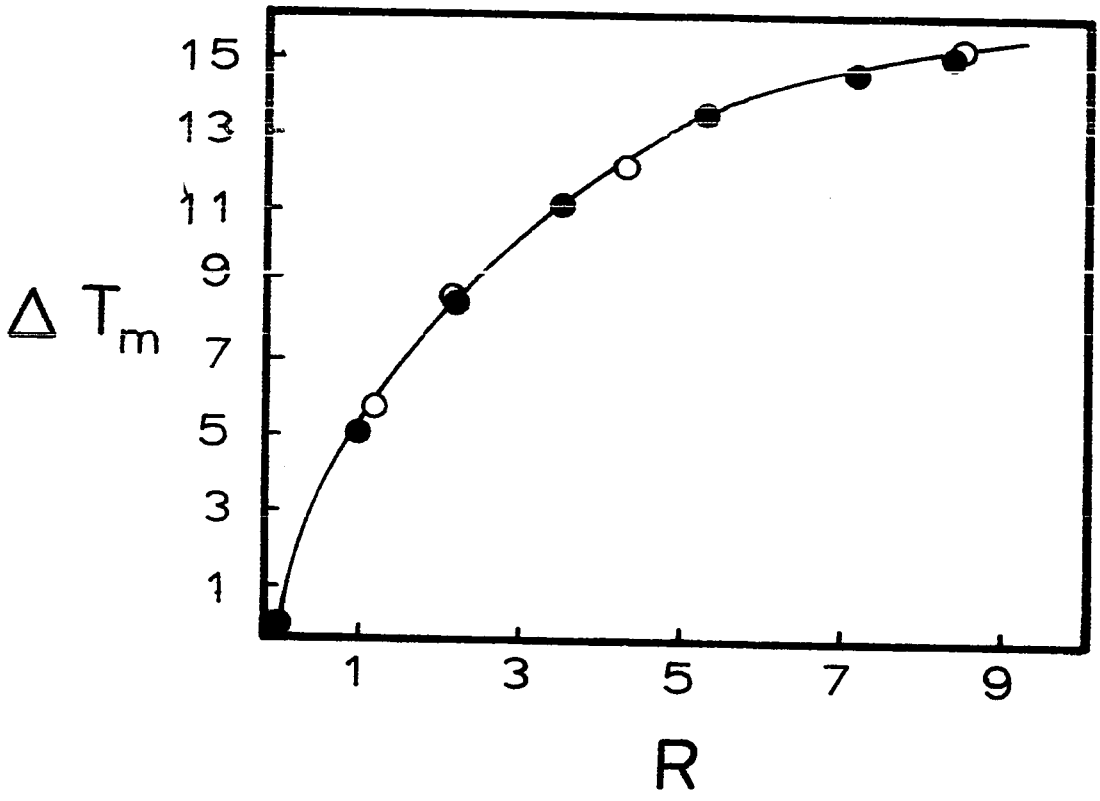
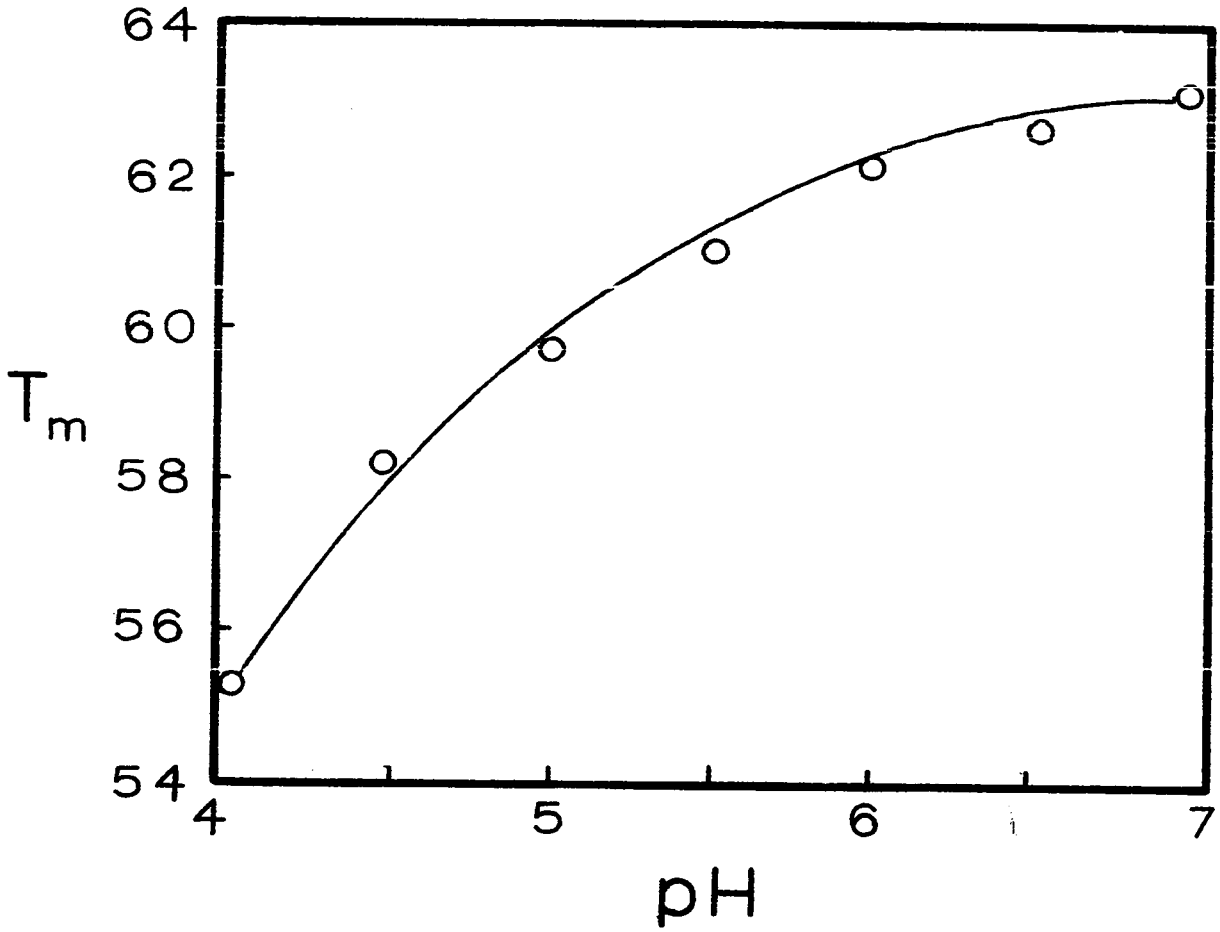

15
13
11
9
7
5
3
1
Δ Tm
1
3
5
7
9
R

FIGURE 25

The transition temperature of RNase as a function of pH in 0.1 M KCl.


64
62
60
58
56
54
T_m
4
5
6
7
pH

imidazole groups has a negligible effect on protein stability as compared to the protonation of carboxyl groups (Hermans and Scheraga, 1961 b), since the curve is fairly flat in the pH range above 6.0.

The results of thermal denaturation in 0.1 M KCl over a pH range of 4.5 to 7.0 and an R range of 1 to 9 are shown in Figure 26 as a plot of the difference in T_m for the metal:protein complex compared to RNase itself. The curves are somewhat irregular in shape and show an anomaly at high pH and low R. This anomaly is more obvious if the same data plus results at additional R values, are presented at constant pH as a plot of ΔT_m vs R. This plot is Figure 27.

There is a difficulty in interpreting Figures 26 and 27 because one cannot interpret the variations in a stability parameter such as T_m as being due just to changes in R when the pH is also being changed. R is not a measure of the amount of metal ion bound to produce Cu(II):RNase, and values of T_m compared at different pH values must be corrected to an equivalent state of binding. In lieu of a direct measure of $\bar{\nu}$, metal ions bound per molecule of protein, Table 2 may be used to allow a better presentation of the data in Figures 26 and 27. To produce Figure 28, a function, ϕ, of the extent of binding is defined as the ratio of the protons displaced by metal ion at pH 7.0 to protons displaced at pH 5.5 at a given value of R. Values of ΔT_m observed at pH 5.5 are then multiplied by ϕ;

FIGURE 26

The effect of Cu(II) on T_m at constant pH. Closed circles; Cu(II) absent from reference cell. Open circles; Cu(II) in reference cell at pH 7.0.

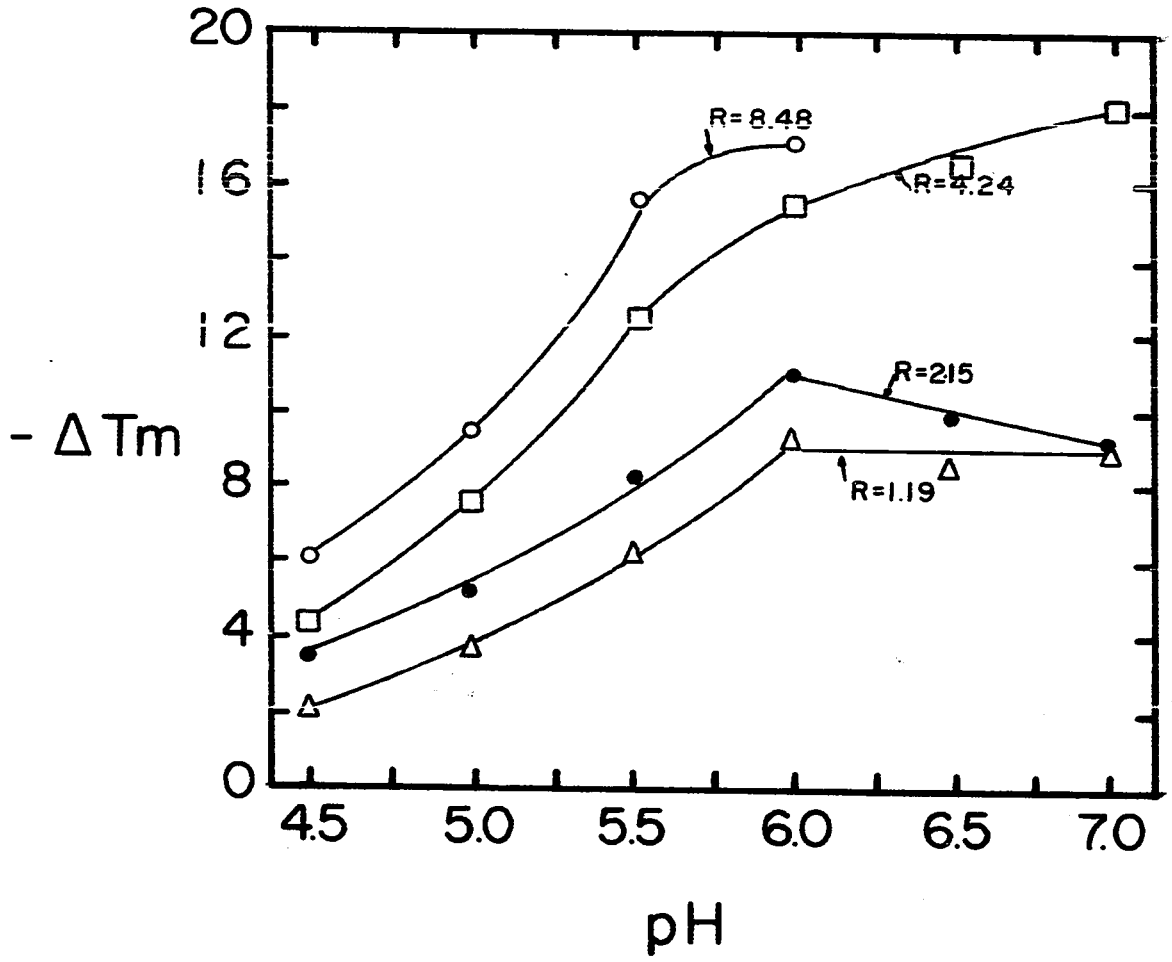

20
16
12
8
4
0
- ΔTm
R=8.48
R=4.24
R=2.15
R=1.19
4.5
5.0
5.5
6.0
6.5
7.0
pH

FIGURE 27

The effect of Cu(II) on T_m at constant R.

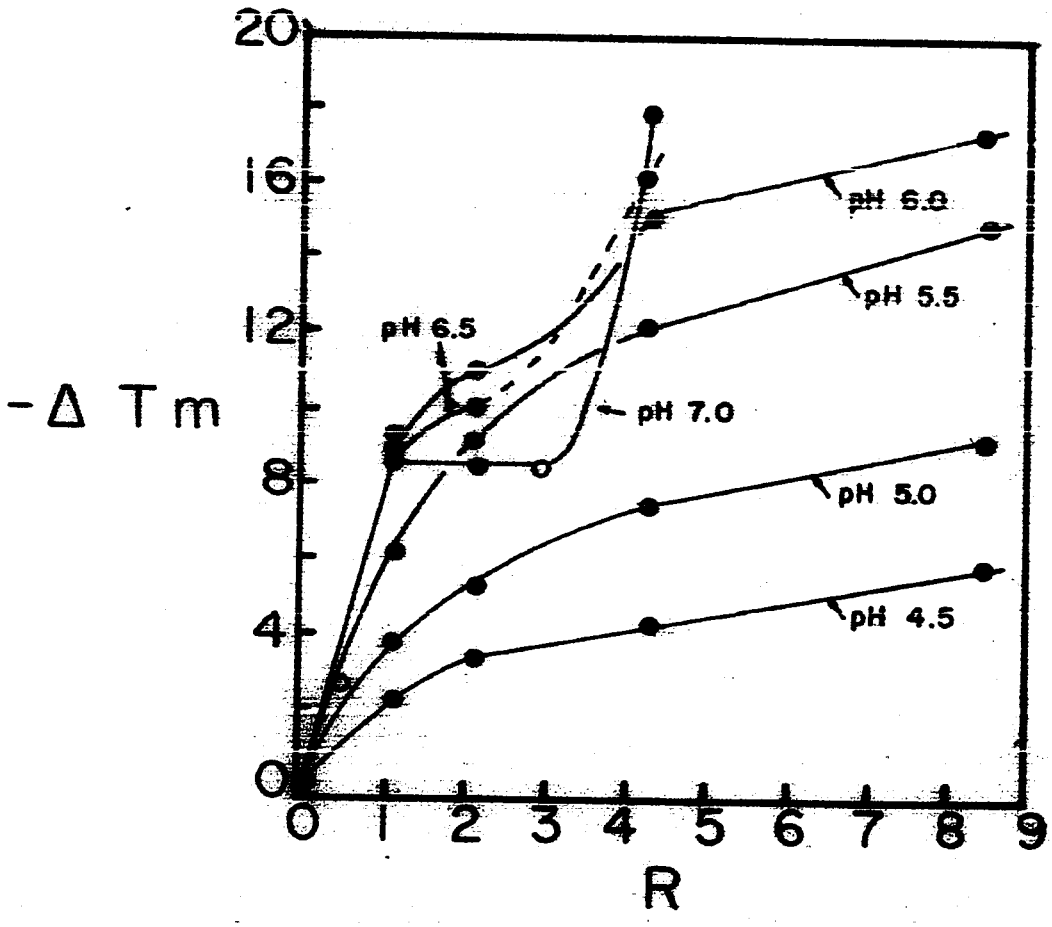
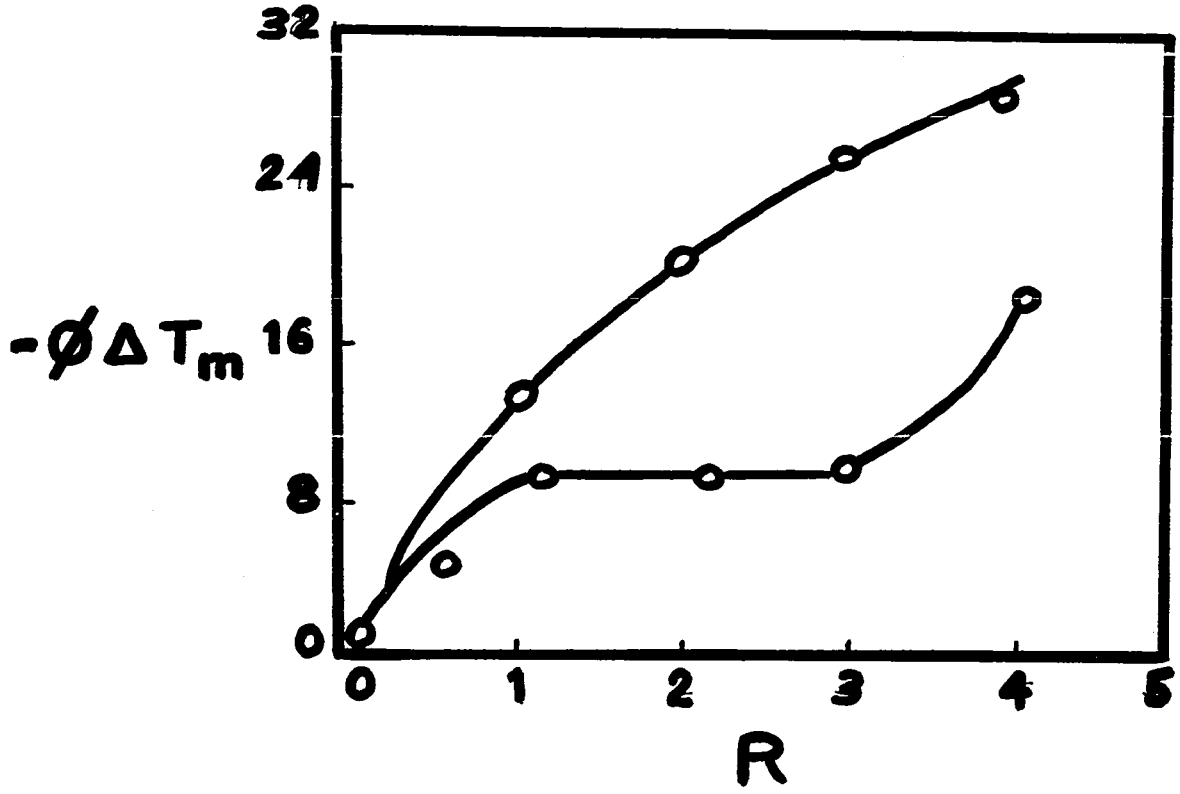

20
16
12
8
4
0
-Δ Tm
0 1 2 3 4 5 6 7 8 9
R
pH 6.5
pH 7.0
pH 6.0
pH 5.5
pH 5.0
pH 4.5

FIGURE 28

The effect of Cu(II) on T_m at pH 5.5 and 7.0.

Corrected for the extent of binding.

The bottom curve shows data at pH 7.0.


32
24
16
8
0
$-\phi\Delta T_m$
0
1
2
3
4
5
R

ΔT_m values at pH 7.0 remain unchanged because ϕ is unity whereas ΔT_m values observed at pH 5.5 are normalized to the state of binding which exists at pH 7.0. R values greater than 4 are not included in the calculations which produced Figure 28 because at pH 7.0 such R values overlap into the area of irreversible titration shown in Figure 14. This is important from considerations other than the reversibility of titration since the release of protons at values of R and pH which fall above the dotted line in Figure 14 is due to the formation of a second set of binding sites and the extent of binding correction, ϕ, is valid for the purpose of Figure 28 when the histidyl-containing binding sites only are considered.

Figures 26, 27 and 28 taken together show clearly that there is a reversal in the destabilizing effect of Cu(II) on RNase at pH values greater than 5.5 as more than one binding site becomes occupied. Therefore, the four histidyl-containing binding sites, identified by Breslow and Girotti (1966) must be considerably different in their local environment at pH 7.0, and appear to be occupied by Cu(II) in some sort of sequential fashion. At pH 5.5, the four binding sites are again seen not to be conformationally equivalent, since each of them when occupied by Cu(II) appears to vary in their effect on RNase stability but the smoothness of the pH 5.5 curve in Figure 28 suggests that occupancy of the sites occurs in a more random statistical manner rather than sequentially.

E. Denaturation of 1-carboxymethylhistidyl-119-RNase

The results and discussion of thermal denaturation experiments carried out with this derivative are given in Appendix 2.

F. Kinetics of Formation of Carboxymethylated Ribonucleases

The results of ion exchange separation of the components of the carboxymethylation reaction mixture in the absence of Cu(II) are shown in Figure 29. Crestfield et al (1963c) have shown that this reaction is first order kinetically with respect to protein and to iodoacetate so that the overall second order kinetic rate can be expressed as:

$$-\frac{d(\text{RNase})}{dt} = k\,(\text{RNase})\,(\text{Iodoacetate}) \qquad (6)$$

Using the results shown in Figure 29, it is possible to compare the rate of reaction observed in this work with that reported by Crestfield et al (1963 b, c). Figure 30 shows the good agreement between these two sets of data and it is concluded that the Mann RNase A used in the carboxymethylation experiments produced the expected kinetic rate, as well as the expected ratio of products. The points plotted in Figure 30 were calculated from simple plots of the amounts of RNase, 1-carboxymethylhistidyl-119-ribonuclease and 3-carboxymethylhistidyl-12-ribonuclease present at any time using the data from Figure 29 and the chromatograms of Crestfield et al (1963c). Using equation 6, corrections were made on the data shown in Figure 29 to normalize them to the same initial concentrations used by Crestfield et al (1963c). To facilitate the calculations the maximum value of the absorbance at the peak height rather than the

FIGURE 29

Chromatograms obtained at different times during the alkylation of RNase. An aqueous solution 1% in RNase and 0.6% in iodoacetate was maintained at 25° and pH 5.5. Samples were withdrawn periodically, diluted 2:1, and 250 µl were applied to the BioRex 70 column for analysis.

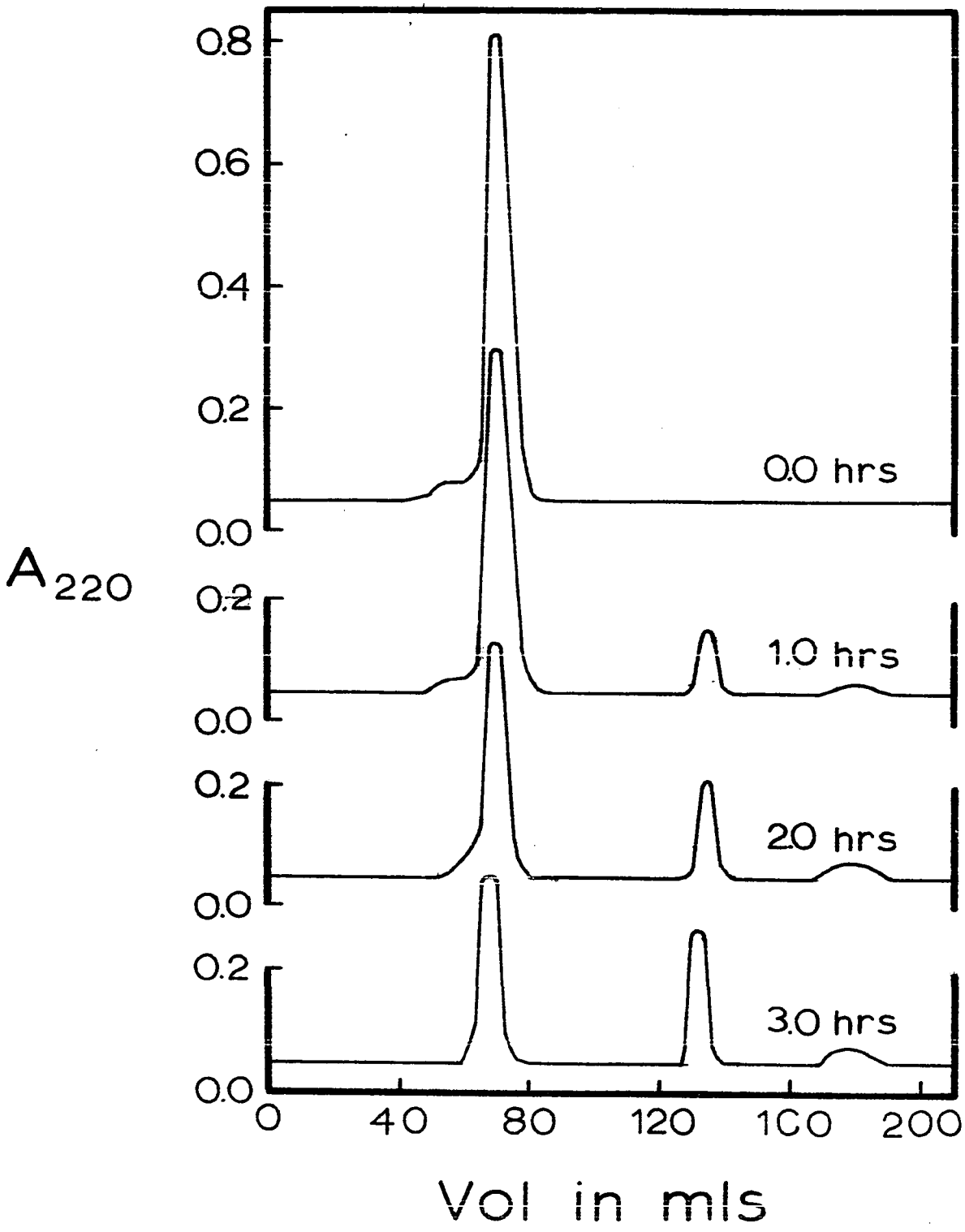
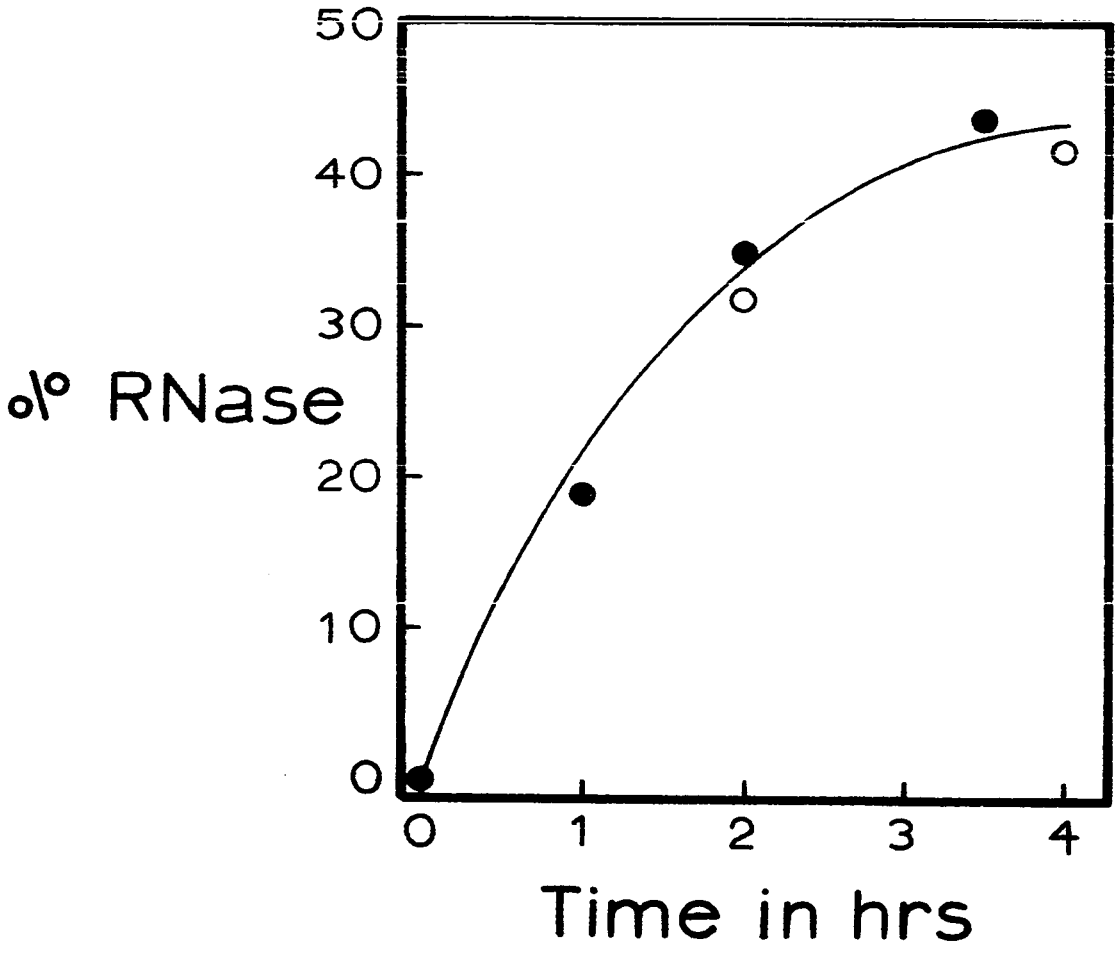

0.8
0.6
0.4
0.2
0.0
0.0 hrs
A220
0.2
0.0
1.0 hrs
0.2
0.0
2.0 hrs
0.2
0.0
3.0 hrs
0
40
80
120
160
200
Vol in mls

FIGURE 30

The rate of product formation during alkylation of RNase by iodoacetate at 25° and pH 5.5.

Closed circles; data from this work.

Open circles; data from Crestfield et al (1963 c).


50
40
30
20
10
0
% RNase
0
1
2
3
4
Time in hrs

area under the peak in Figure 30 was taken to be proportional to concentration; this is valid as long as peak symmetry is observed. It has been shown by Gundlach et al (1959) and by Crestfield et al (1963 b, c) that only the two derivatives noted above are formed at pH 5.5 in the reaction with iodoacetate at a ratio of 1-carboxymethylhistidyl-119-ribonuclease to 3-carboxymethylhistidyl-12-ribonuclease of 8 to 10.

The overall kinetic rate for alkylation of RNase by iodoacetate in the presence of Cu(II) as determined using the pH-stat, was found to be pseudo-first order due to the large excess of iodoacetate. This large excess was necessary to allow a measurable rate of proton release and under these conditions equation 6 may be written as:

$$- \frac{d(\text{RNase})}{dt} = k\,(\text{RNase}) \qquad (7)$$

or as

$$\frac{dx}{dt} = k\,(a - \alpha) \qquad (8)$$

where a is the initial concentration of RNase and x is the decrease in concentration of RNase at time t. Equation 8 may be integrated to give

$$2.303 \log \frac{a}{a - x} = k\,t \qquad (9)$$

and plots of log (a/a - x) against t should be linear with a slope equal to the rate constant k divided by 2.303.

Figure 31 shows plots of the kinetic data obtained at pH 5.5, plotted according to equation 9. Figure 32 is a similar plot of the data obtained at pH 7.0. It can be seen that equation 9 is being obeyed at both pH values. However, there is some random error present in the data presented in either Figure 31 or Figure 32. The random error shown is probably a result of small temperature variations permitted by the Haake circulating pump as well as of the low concentration of the base, 0.0195M, used in the syringe of the pH-stat. The pH-stat was well grounded and showed maximum stability while measuring the pH of standard buffers. Because of the random error observed in these measurements, the solid lines in Figures 31 and 32 were calculated by the method of least mean squares according to the linear correlation procedure presented by Croxton (1953).

Figure 33 shows a plot of the rate constants as a function of R determined at pH 5.5 from the slopes of the lines in Figure 31. Figure 33 indicates that saturation of one binding site results in the complete inhibition of carboxymethylation of RNase, since inhibition is very nearly complete at an R value of 2, i.e., when one metal ion is bound per molecule of RNase since pH 5.5 is the pH of half-binding. Since inhibition is essentially complete when one Cu(II) is bound, these results appear to contradict the conclusion drawn from the thermal denaturation data that occupancy of the Cu(II)

FIGURE 31

Pseudo 1st-order kinetic plots from the alkylation of RNase by iodoacetate in the presence of Cu(II) at pH 5.5.

R values from top to bottom are: 0.0, 0.5, 1.0, 2.0, 7.0.

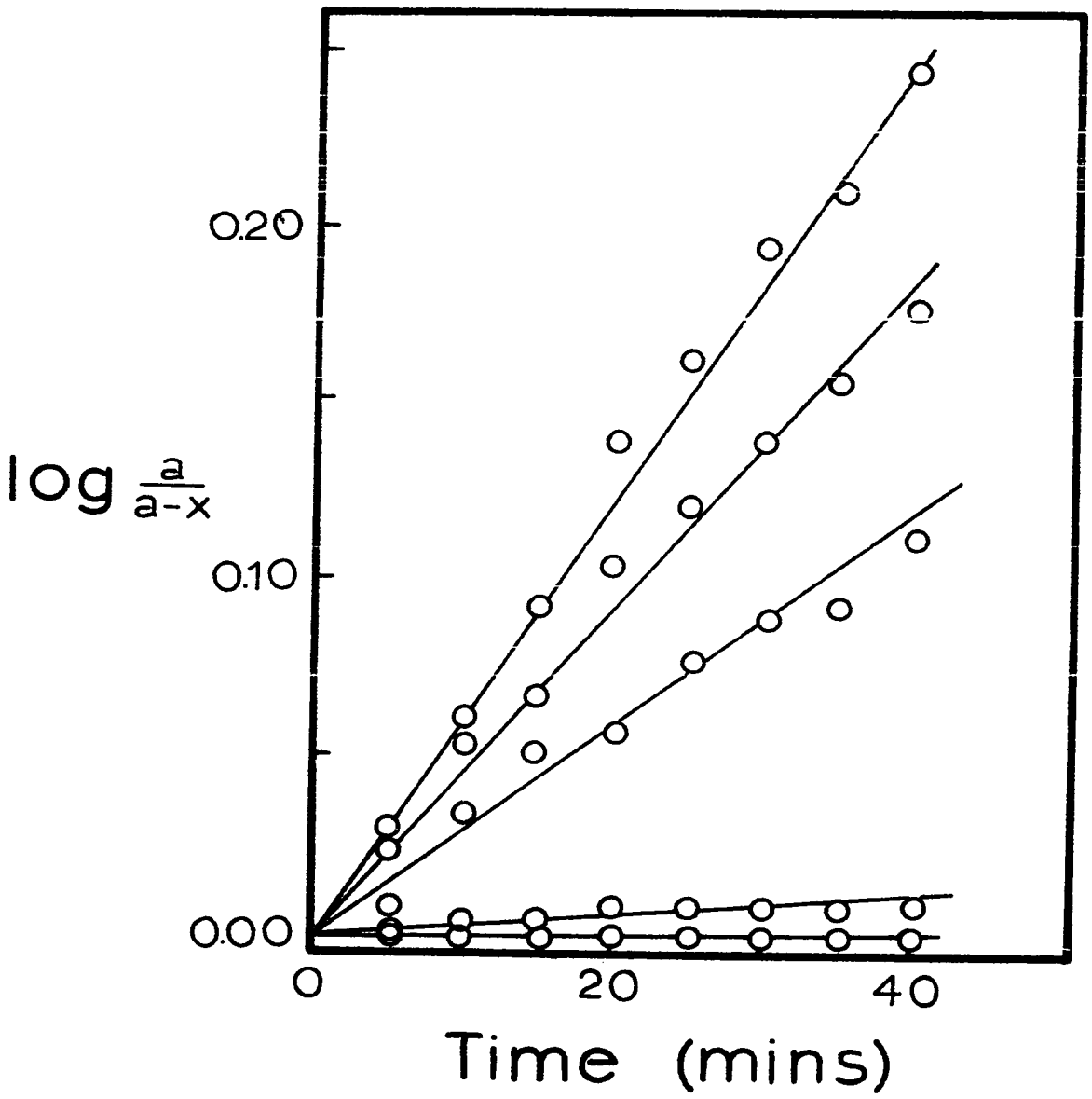

0.20
0.10
0.00
log a/a-x
0
20
40
Time (mins)

FIGURE 32

Pseudo 1st-order kinetic plots for the alkylation of RNase by iodoacetate in the presence of Cu(II) at pH 7.0.

R values from top to bottom are; 0.0, 1.0, 2.0, 3.0, 4.0.

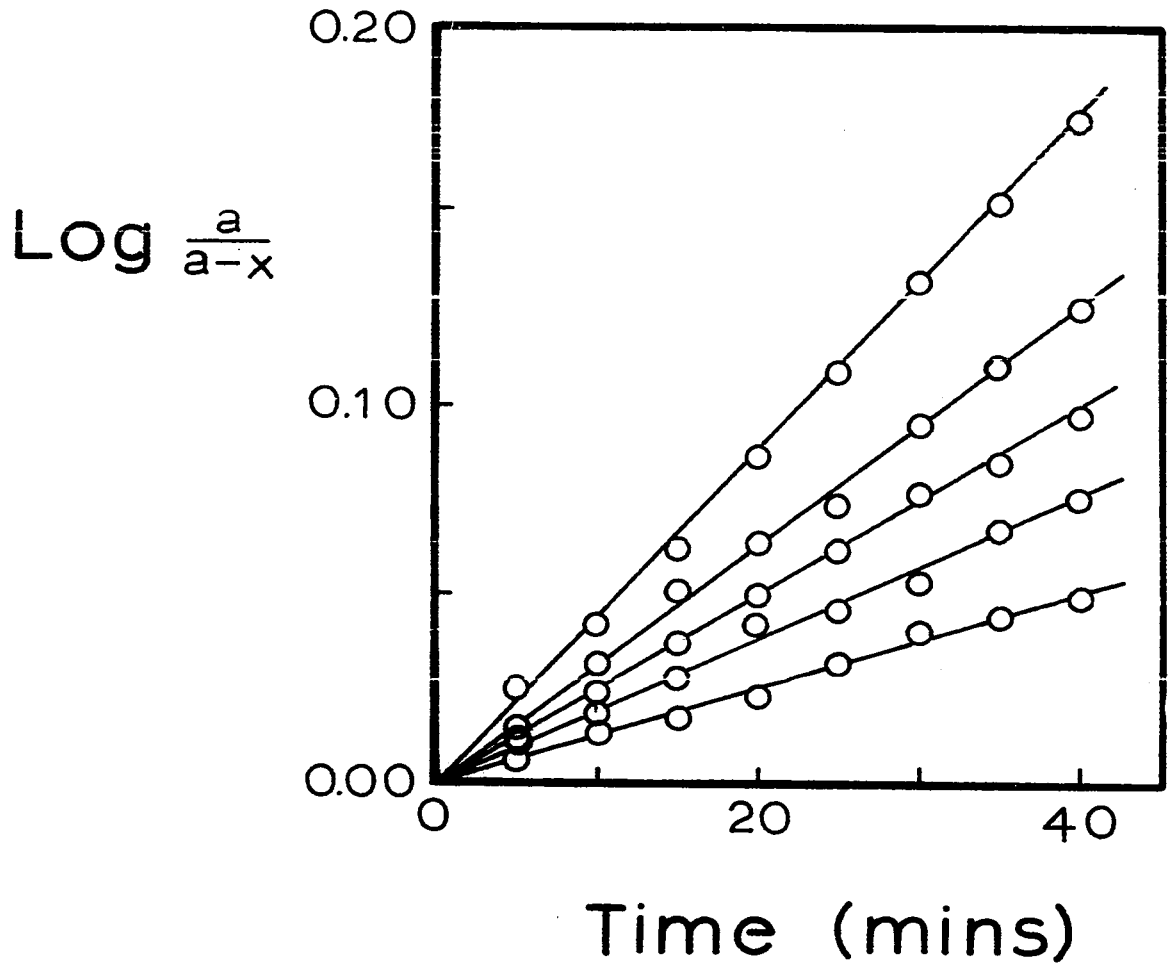
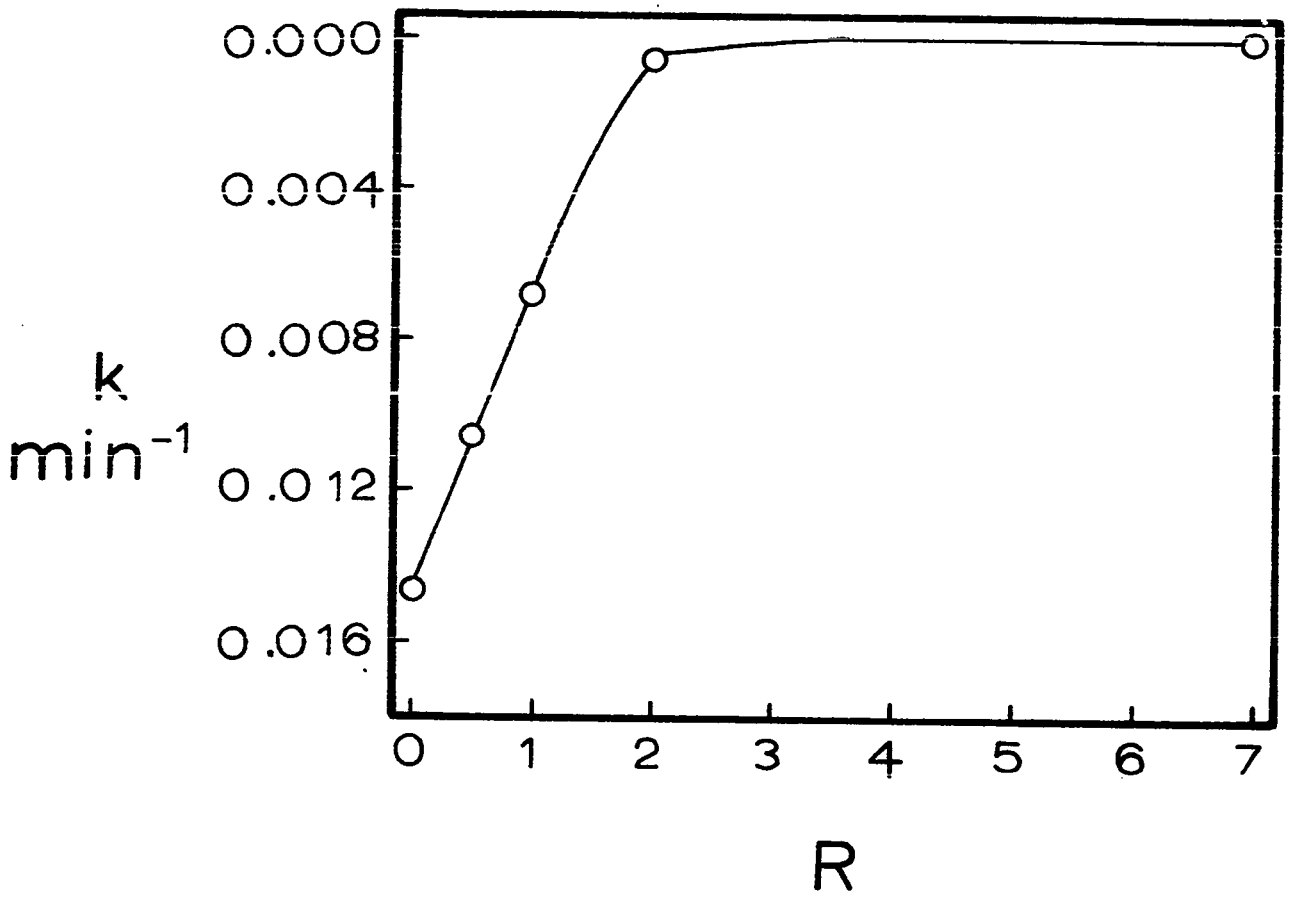

0.20
0.10
0.00
0
20
40
Log a/a−x
Time (mins)

FIGURE 33

The effect of Cu(II) on the rate constant, k, for alkylation of RNase at pH 5.5.


0.000
0.004
0.008
0.012
0.016
k
min⁻¹
0
1
2
3
4
5
6
7
R

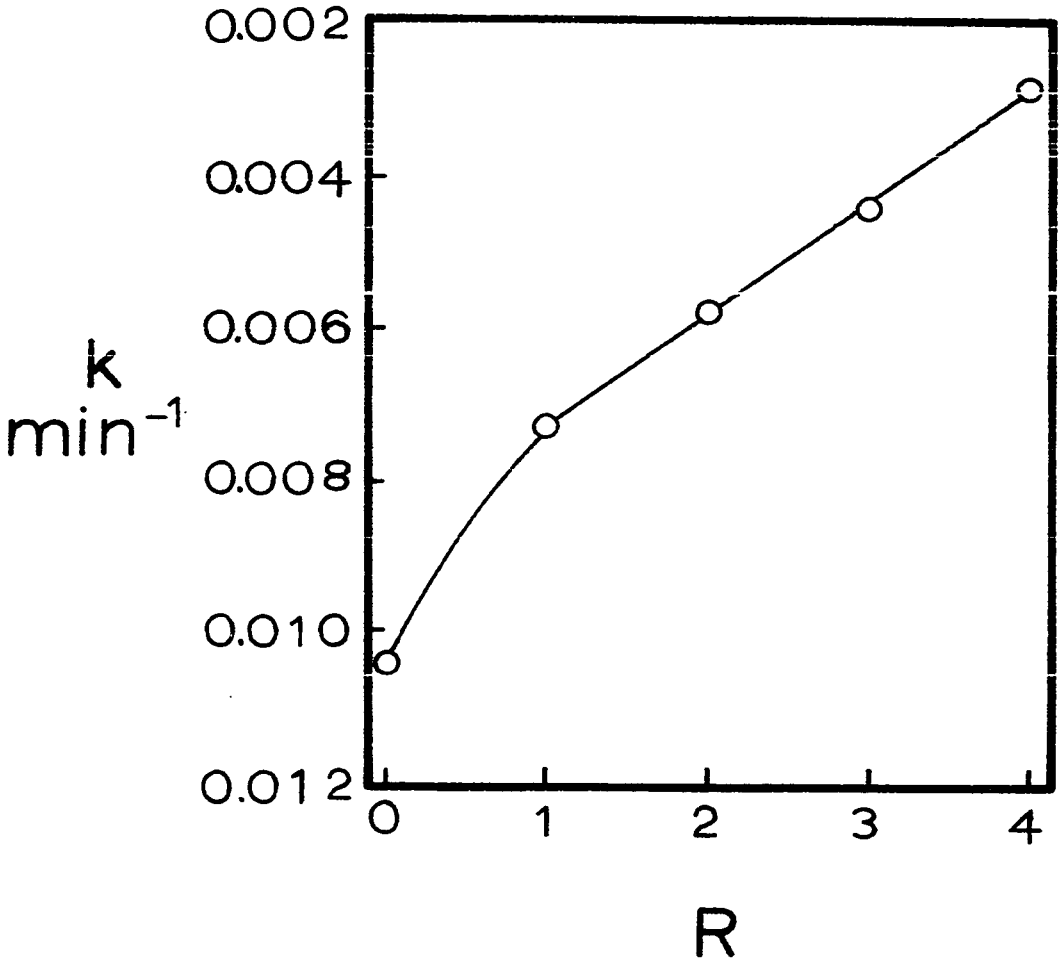
binding-sites at pH 5.5 proceeds in a random statistical manner. This conflict may be resolved by Breslow's (1967) finding that the first binding site at pH 5.5, the site found to inhibit carboxymethylation according to Figure 33, has an association constant one order of magnitude larger than that of the association constants for the next four sites. Thus, the first binding-site only may be considered to be preferentially saturated.

The experimental point at an R value of 7 is included in the data presented in Figure 33 because this is the value of R at which Crestfield et al (1963c) found Cu(II) to inhibit almost completely the formation of 1-carboxymethyl-119-ribonuclease while having only a small effect on the formation of 3-carboxymethyl-histidyl-12-ribonuclease. Figure 33 indicates that 100% inhibition of the alkylation of RNase by iodoacetate at pH 5.5 occurs at an R value of 7, under the conditions employed in this work.

Figure 34 shows the effect of Cu(II) on the rate constant for carboxymethylation of RNase at pH 7.0. The results in this case do not show complete inhibition of the carboxymethylation reaction at any value of R at which measurement was made. Again no measurements were made at pH 7.0 with values of R which would produce binding of Cu(II) to sites exhibiting irreversible titration behavior. An R value of 3.5 at pH 7.0 is equivalent to an R value of 7.0 at pH 5.5. The results shown in Figures 33 and 34

FIGURE 34

The effect of Cu(II) on the rate constant, k, for alkylation of RNase at pH 7.0.


0.002
0.004
0.006
0.008
0.010
0.012
k
min⁻¹
0
1
2
3
4
R

reinforce the conclusion from the thermal denaturation data that the binding of Cu(II) at pH 5.5 is both qualitatively and quantitatively different from the binding observed at pH 7.0.

Photographs of electrophoretic separations of samples from each of the carboxymethylation reaction mixtures are shown in Figures 35 and 36. Figure 35 shows samples from reactions run at pH 5.5; Figure 36 shows the separation obtained from samples of reactions run at pH 7.0. The electropherograms obtained from the pH 5.5 reaction mixture show two distinct bands which may be attributed to RNase itself and to the carboxymethylated derivatives. The top photograph in Figure 35 shows the results of electrophoresis of a sample of RNase from the stock solution of protein. From this electropherogram it is possible to identify the most rapidly migrating band on the remainder of the strips as RNase; the slower band must then be attributed to the derivatives. Figure 36 shows the presence of a third minor component which migrates at a rate slower than that attributed to the two carboxymethylhistidyl derivatives. The minor component could be a carboxymethylysyl derivative since Gundlach et al (1959) concluded that reaction at pH 8.5 with iodoacetate resulted in the formation of ε-carboxymethyllysyl-41-ribonuclease. However, it might reasonably be expected that larger amounts of this derivative than form the minor band of Figure 36 might be expected to form at pH 7.0 if the derivative is formed exclusively at pH 8.5.

FIGURE 35

Sepraphore III strips illustrating electrophoretic separations from samples of carboxymethylation reaction mixtures at pH 5.5. R values are noted below each strip.

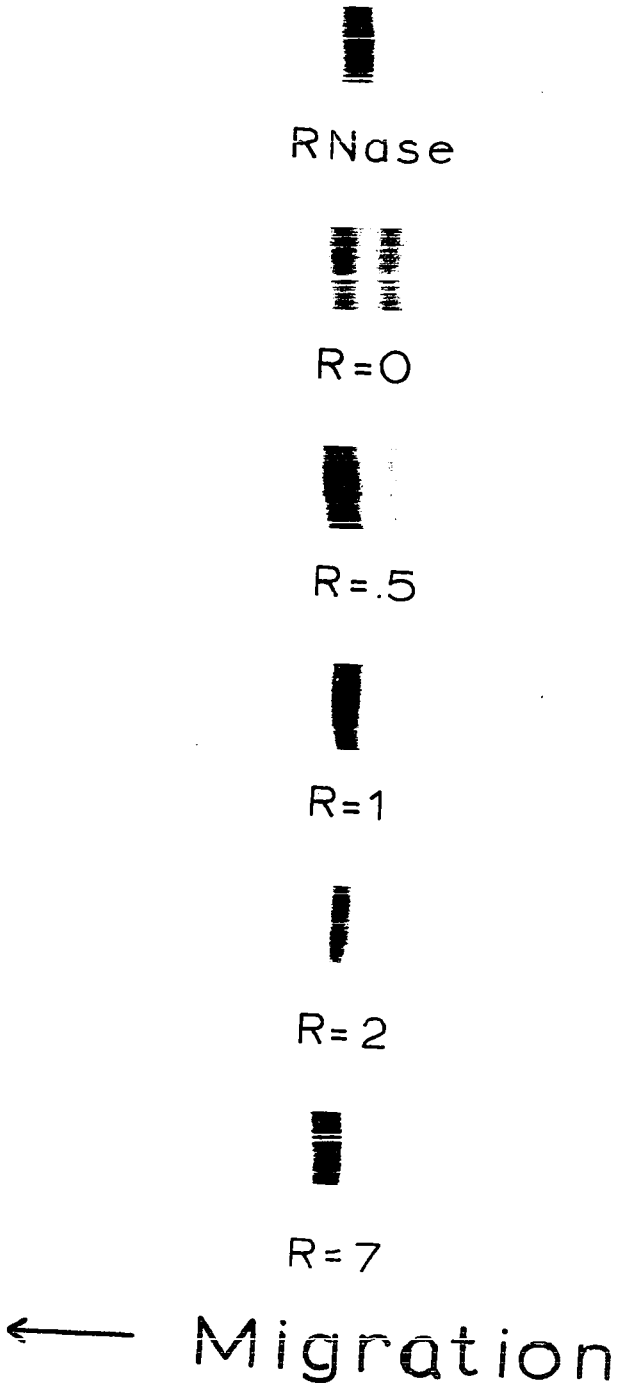

RNase
R=0
R=.5
R=1
R=2
R=7
Migration

FIGURE 36

Sepraphore III strips illustrating electrophoretic separations from samples of carboxymethylation reaction mixtures at pH 7.0. R values are noted below each strip.

R = 0

R = 1

R = 2

R = 3

R = 4

← Migration

Figure 37 shows the results of densitometer recordings for each of the reaction mixtures studied electrophoretically. Although the best three electropherograms from each reaction mixture were quantitated, only the best result is shown in Figure 37. The results obtained electrophoretically are shown in Figures 38 and 39 as plots of the percent inhibition observed vs R at pH 5.5 and 7.0 respectively. By comparison with Figures 33 and 34, it can be seen that the agreement with the pH-stat determinations ranges from excellent to fair.

These electrophoretic determinations will not be discussed further, but they are included in some detail because they represent one of the few known attempts to use electrophoresis to separate RNase and its derivatives. The use of the method was suggested by the work of Brown (1963), who showed that the electrophoretic migration rates of RNase and 1-carboxymethylhistidyl-119-ribonuclease are distinguishable as would be expected from considerations of net charge. It is possible that the electrophoretic method could be developed further to yield a high accuracy.

G. Quantitation

1. Denaturation Reaction Order

In Figure 23, the logarithm of the equilibrium constant for denaturation of RNase in urea solution was plotted against the logarithm of R. The line drawn in this figure was drawn purposely with

FIGURE 37

Densitometer recordings of electropheretic separations of carboxymethylation reaction mixtures in the presence of Cu(II). R values appear below each recording.

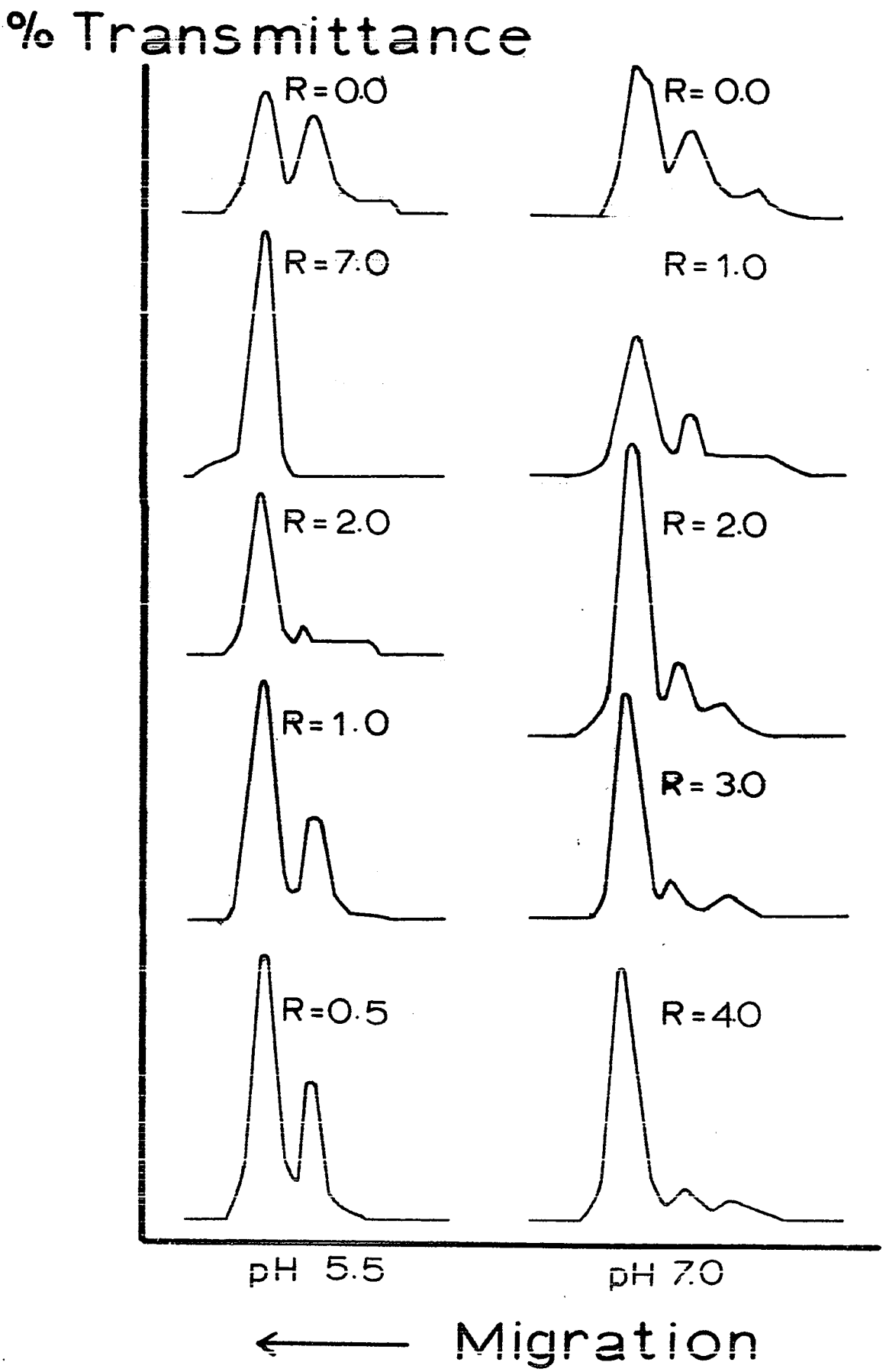

% Transmittance
R=0.0
R=7.0
R=2.0
R=1.0
R=0.5
R=0.0
R=1.0
R=2.0
R=3.0
R=4.0
pH 5.5
pH 7.0
Migration

FIGURE 38

Percent inhibition by Cu(II) of the carboxymethylation of RNase at pH 5.5 as observed electrophoretically.

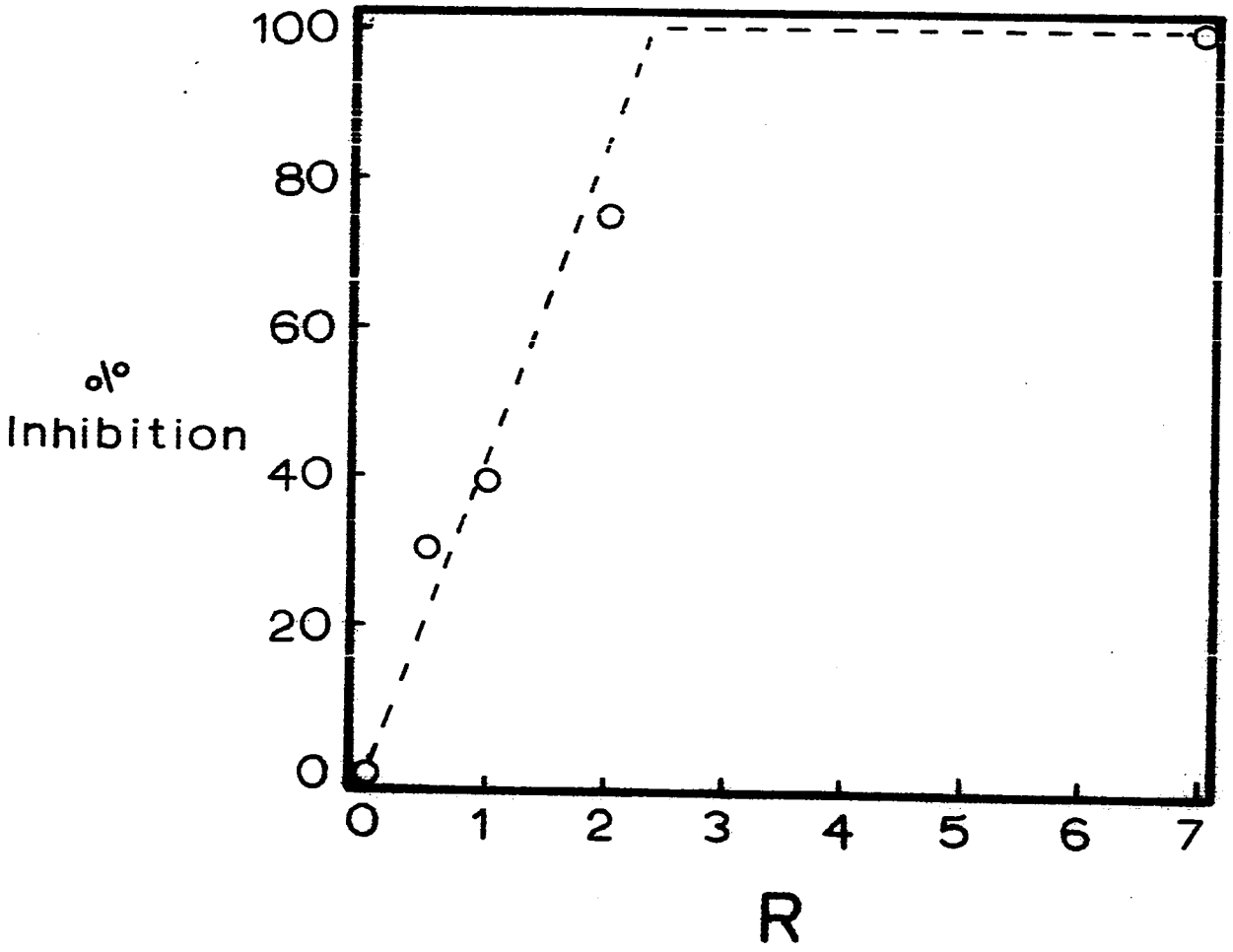
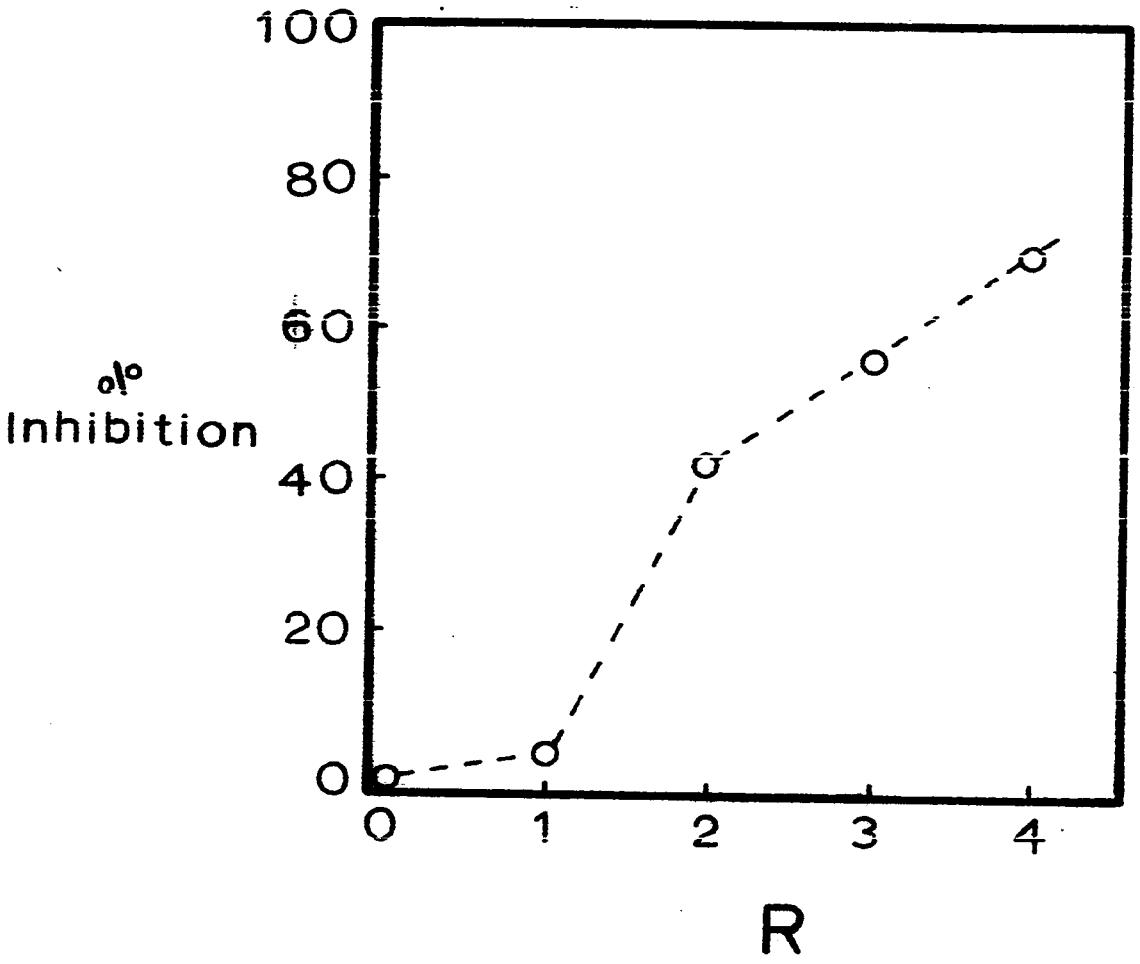

100
80
60
40
20
0
% Inhibition
0
1
2
3
4
5
6
7
R

FIGURE 39

Percent inhibition by Cu(II) of the carboxymethylation of RNase at pH 7.0 as observed electrophoretically.


100
80
60
40
20
0
% Inhibition
0
1
2
3
4
R

a slope of unity, although this slope does provide the best fit of the data. In this section, similar plots are presented for thermal denaturation data for the purpose of determining the number of Cu(II) binding sites which contribute to the destabilization of RNase at a particular pH. Plots of this type are encountered frequently in the literature and Appendix 3 gives a derivation resulting in the equation:

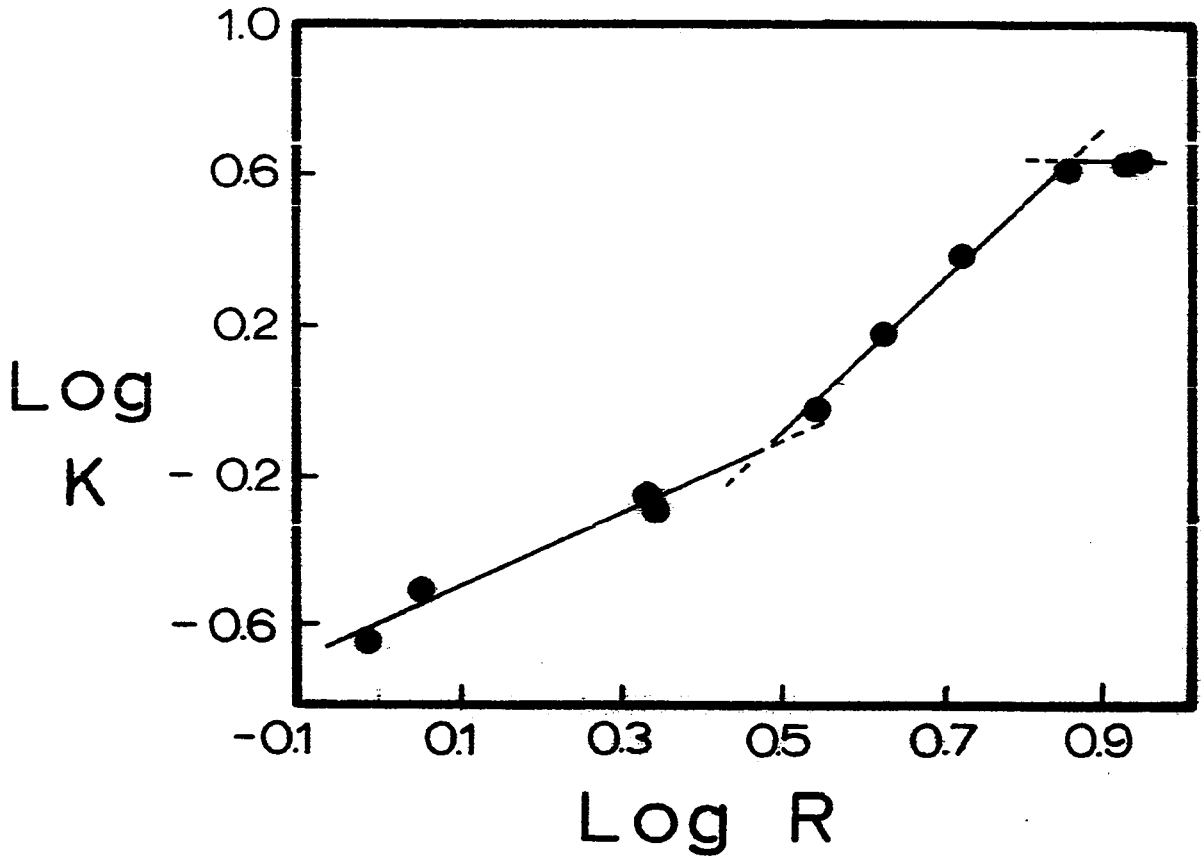
$$\log K = n \log R + n \log \left\{ [\text{RNase}] \quad (1 - \bar{r}) \right\} + C \qquad (10)$$

Equation 10 justifies the log K vs log R plot so long as the limitations on its use given in Appendix 3 are observed. In equation 10, n refers to the number of Cu(II) participating in destabilization and is equal to the slope.

If the data illustrated in Figure 24 are plotted as log K vs log R, the results are as shown in Figure 40 where the equilibrium constant values were calculated at 50°. Figure 40 is divided into three sections, each giving a different value for the reaction order, i.e., the value of n. Two points of intersection then appear. The four points below the transition midpoint at log K equal to zero give an apparent reaction order of unity and a line through these points intersects with the line from the data immediately above the transition midpoint. This point of intersection turns out to correspond quite closely to the value of log K at which Hermans and Scheraga (1961a) found the enthalpy of denaturation for RNase to change its value at pH 5.5. The data above the transition midpoint give a

FIGURE 40

Reaction Order Plot for the effect of Cu(II) on thermal denaturation of RNase at 50°C, pH 5.55.


1.0
0.6
0.2
- 0.2
- 0.6
Log K
-0.1
0.1
0.3
0.5
0.7
0.9
Log R

reaction order of 2 and intersect with the remaining data at values of R at which destabilization of the protein is complete.

If the slopes in Figure 40 are taken to be valid measures of the reaction order, then the number of binding sites involved in destabilization are respectively 1, 2, and 0. The latter value is self-evident but the abrupt change in the reaction order from 1 to 2 near the midpoint of transition probably means that an additional Cu(II) is involved in a second phase of thermal denaturation. If this interpretation is true, then plots similar to Figure 40 but done at higher and lower temperatures than 50° should give the lower intersection point at the same value of log K but with increasingly more or fewer, respectively, experimental points falling on the line denoting a reaction order of 2. This is expected since it is the heat energy, not the Cu(II), that is primarily responsible for the denaturation process. Figures 41 and 42 show plots of data obtained at temperatures of 47.5° and 52.5°. It is seen that the lower intersection point does remain at about the same value of log K with the predicted change in positioning of the points being observed. The lines in Figures 40, 41 and 42 are drawn with slopes of 1.0, 2.0 and 0.0.

The reaction order observed in urea, as shown by Figure 20, remains constant at unity over the same range of values of R shown in Figure 40 at the same pH.

FIGURE 41

Reaction order plot for the effect of Cu(II) on thermal denaturation of RNase at 47.5°C, pH 5.55.

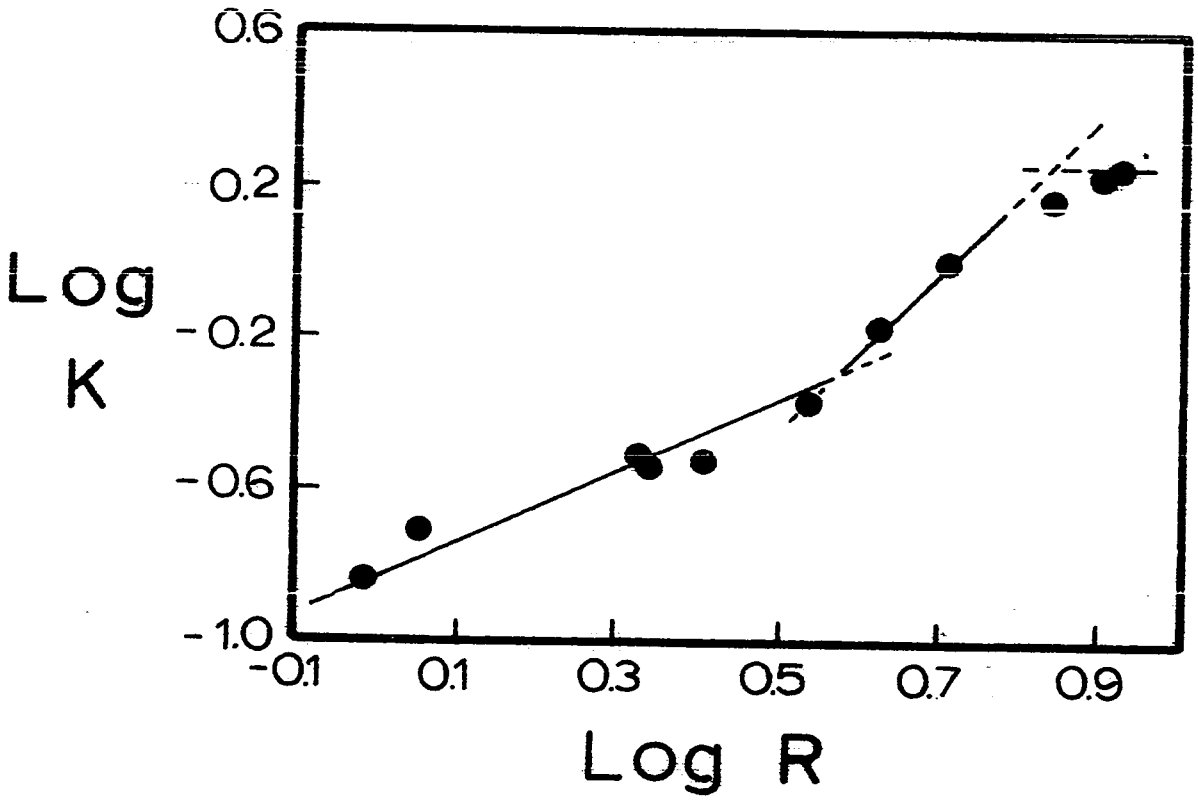
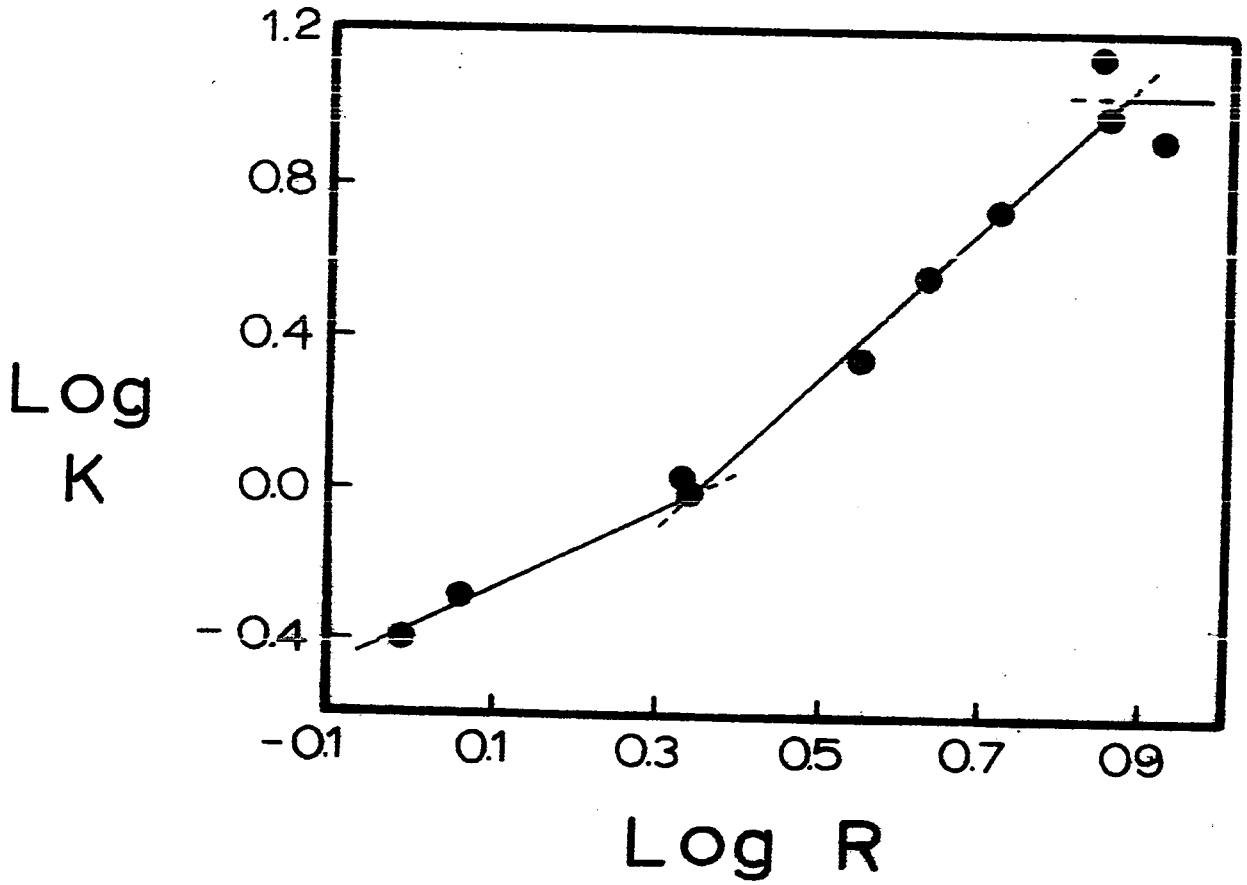

0.6
0.2
-0.2
-0.6
-1.0
Log K
-0.1
0.1
0.3
0.5
0.7
0.9
Log R

FIGURE 42

Reaction order plot for the effect of Cu(II) on thermal denaturation of RNase at 52.5°C, pH 5.55.


1.2
0.8
0.4
0.0
-0.4
Log K
-0.1
0.1
0.3
0.5
0.7
0.9
Log R

Reaction order determinations are not possible for Cu(II) destabilization of RNase at pH 7.0 due to the apparent sequential binding of the metal ion as evidenced by Figure 28.

2. Reaction Orders for Carboxymethylation of RNase

These are available by inspection from pH 5.5 plot of percent inhibition of carboxymethylation as 1 from R = O to R = 2 and O from thereon. PH 7.0 is indeterminate.

3. Thermodynamic Parameters

Van't Hoff plots of log K vs I/T, where T is the absolute temperature, may be used to calculate thermodynamic parameters for any denaturation process where values of the equilibrium constant are determinable. However, since the thermal denaturation data for RNase in the presence of Cu(II) is subject to possible indeterminancy at lower temperatures (Section IV-B) van't Hoff plots of the data would be really valid only as the values of log K approach zero. In this region, however, van't Hoff plots begin to show curvature (Hermans and Scheraga, 1961a) which persists until log K approaches unity. Therefore, the approach used in this work was to calculate values of the standard enthalpy change for RNase thermal denaturation in the presence of Cu(II) from a modification of the van't Hoff equation which considers only the transition temperature at constant pH. At T_m, the standard free energy change is necessarily zero since the value of the equilibrium constant is unity and

$$\Delta F^{o} = - \mathcal{R} T \ln K \qquad (11)$$

The standard entropy change may then be calculated from a knowledge of the enthalpy and free energy changes.

The equation used for the calculation of the standard enthalpy change, ΔH^o, for thermal denaturation in the presence of Cu(II) is

$$n \ln R = \frac{\Delta H^o}{\mathcal{R} T_m} + Q' \qquad (12)$$

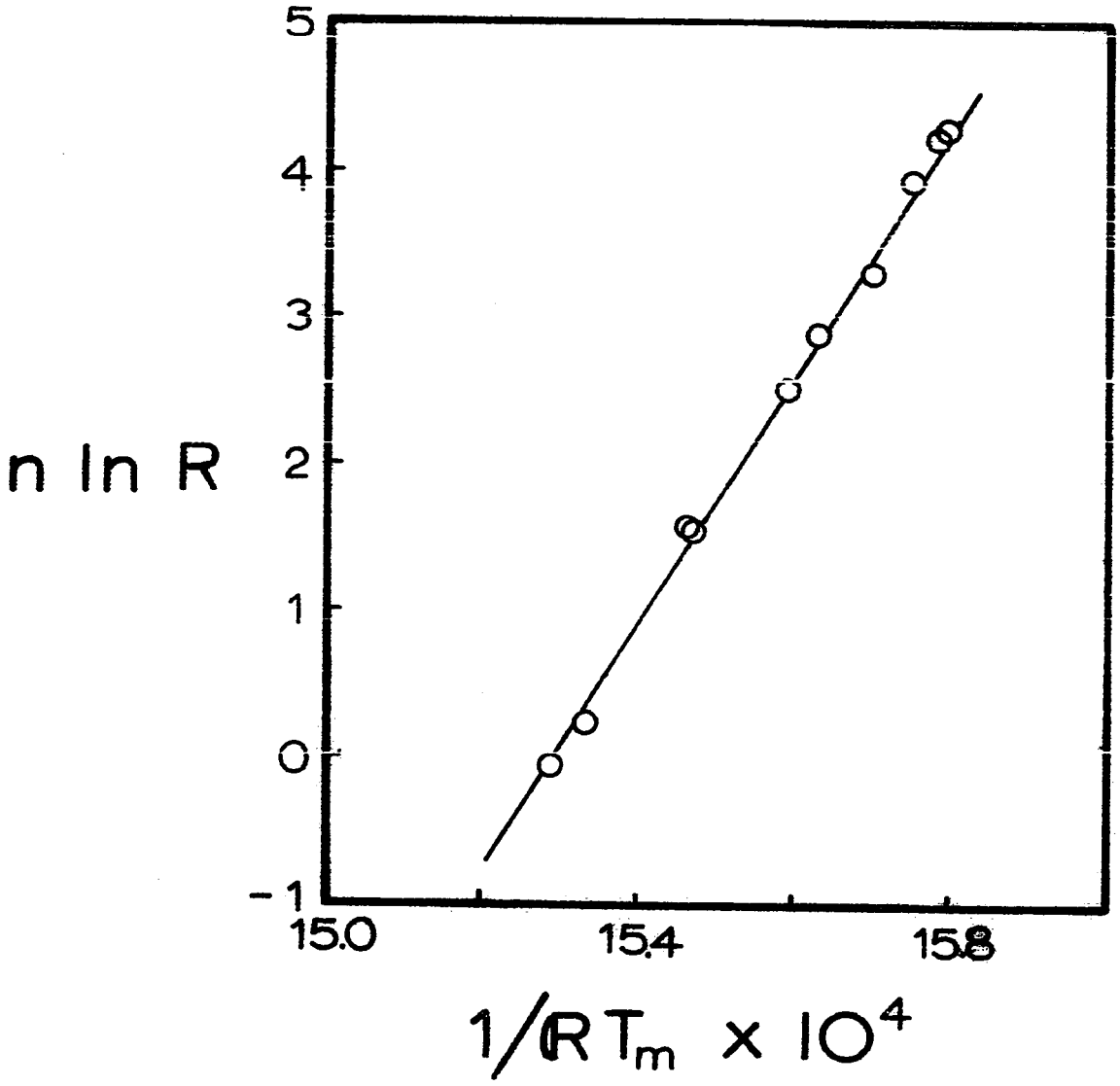
and its derivation is covered in Appendix 3, where it is pointed out that equation 12 provides a linear plot only if destabilization of RNase by Cu(II) does not result in a change in the values of the standard thermodynamic parameters. A linear plot of n ln R vs $1/\mathcal{R}T_m$ is obtained and in shown in Figure 43. The value of n used to produce Figure 43 was 2, since Figures 40, 41 and 42 show that n is equal to 2 at all values of R at the transition temperature. The slope of the line in Figure 43 allows the calculation of ΔH^o. The value obtained is 91 kcal/mole and is in good agreement with the results of Schrier et al (1965) and Hermans and Scheraga (1961a) although the values of ΔH^o obtained by these authors must be estimated from a curved section of a van't Hoff plot. Nevertheless, the agreement is quite good and illustrates the consistency of the methods devised to handle the thermal denaturation data presented in this thesis.

Since the standard enthalpy of denaturation at pH 5.5 in the presence of Cu(II) is constant, the standard entropy, ΔS^o, at the midpoint of transition, may be calculated as

$$\Delta S^o = \Delta H^o / T_m$$

FIGURE 43

Determination of the standard enthalpy for the thermal denaturation of RNase at pH 5.5 in the presence of Cu(II).


5
4
3
2
1
0
−1
n ln R
15.0
15.4
15.8
1/RTm x 10⁴

It is also possible to calculate the difference in the standard entropy, $\Delta(\Delta S^{o})$ (the difference between the standard entropy changes measured in the presence of a given amount of Cu(II), and in its absence). Table 3 presents a summary of the various thermodynamic parameters calculated.

Even though Hermans and Scheraga (1961a) have shown that the standard enthalpy for thermal denaturation of RNase remains constant over the pH range of 2 to 7, all of the calculations above apply only at pH 5.5. This is because of the restriction to random binding processes placed on the use of equation 12 during its derivation in Appendix 3, and because at pH 7.0 random binding is not observed (Figure 28).

TABLE 3

THERMODYNAMIC PARAMETERS FOR THE THERMAL DENATURATION OF RNASE IN THE PRESENCE OF CU(II). ALL VALUES WERE DETERMINED AT THE TRANSITION TEMPERATURE AT PH 5.5

$\Delta F^{o} = 0$

ΔH^{o} = 91 kcal/mole, at all values of R

ΔS^{o} :

R	ΔS^{o}	$\Delta (\Delta S^{o})$
0	272 e.u.	0
0.97	276	4
1.13	277	5
2.15	280	8
2.19	280	8
3.50	281	9
4.24	282	10
5.23	283	11
7.11	285	13
8.36	285	13
8.48	285	13

I. Experiments with Zn(II) Ion

1. Titration Curves

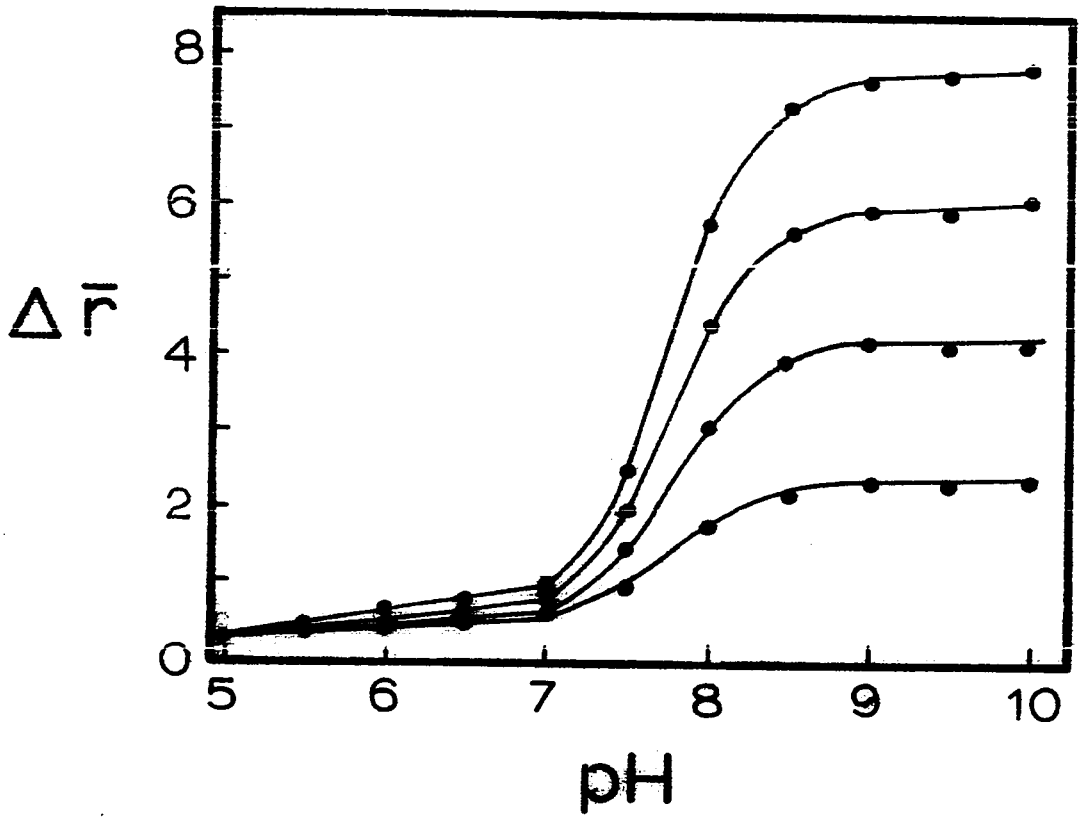
Continuous titration curves were produced for RNase in the presence of Zn(II) at R values from 0 to 4. The results of these experiments are given in Figure 44 as the difference titration curves. If Figure 44 is compared with Figure 5, it is seen that good agreement was obtained with the work of Breslow and Girotti (1966) although the agreement is not quite as good as it was in the case of Cu(II). This is probably because the results shown in Figure 44 were obtained using continuous titration, a procedure observed to give less precise results in this work than did the pH-stat titration method used for titration involving Cu(II). Breslow and Girotti used the continuous titration method for most of their titration experiments but they used larger amounts of protein. All of the titration work reported in this thesis was carried out after it was known that Breslow and Girotti were preparing to publish similar work; the titration experiments were nevertheless required and they were carried out to provide a basic knowledge of the interaction between RNase and the Cu(II) and Zn(II) ions, and to assist in planning denaturation experiments.

Preliminary studies only were carried out on the interaction of Zn(II) with RNase. It was found in the initial stages of this work that Cu(II) was a more convenient ion to work with for the following reasons:

(i) As the pH is increased above 7.0, the denatured complex of RNase and metal ion is increasingly subject to aggregation (Herzig and Bigelow, 1967). With reference to Figure 44, denaturation experiments should be conducted at a pH no

FIGURE 44

Continuous titration curves for RNase in the presence of Zn(II). R values, from top to bottom are: R = 4.0; R = 3.0; R = 2.0; R = 1.0; R = 0.0.


8
6
4
2
0
Δ r̄
5
6
7
8
9
10
pH

lower than 7.7. Cu(II) experiments could be carried out at lower pH values.

(ii) Interpretation of the titration curves produced in the presence of Zn(II) are subject to the uncertainties noted in the Historical Review.

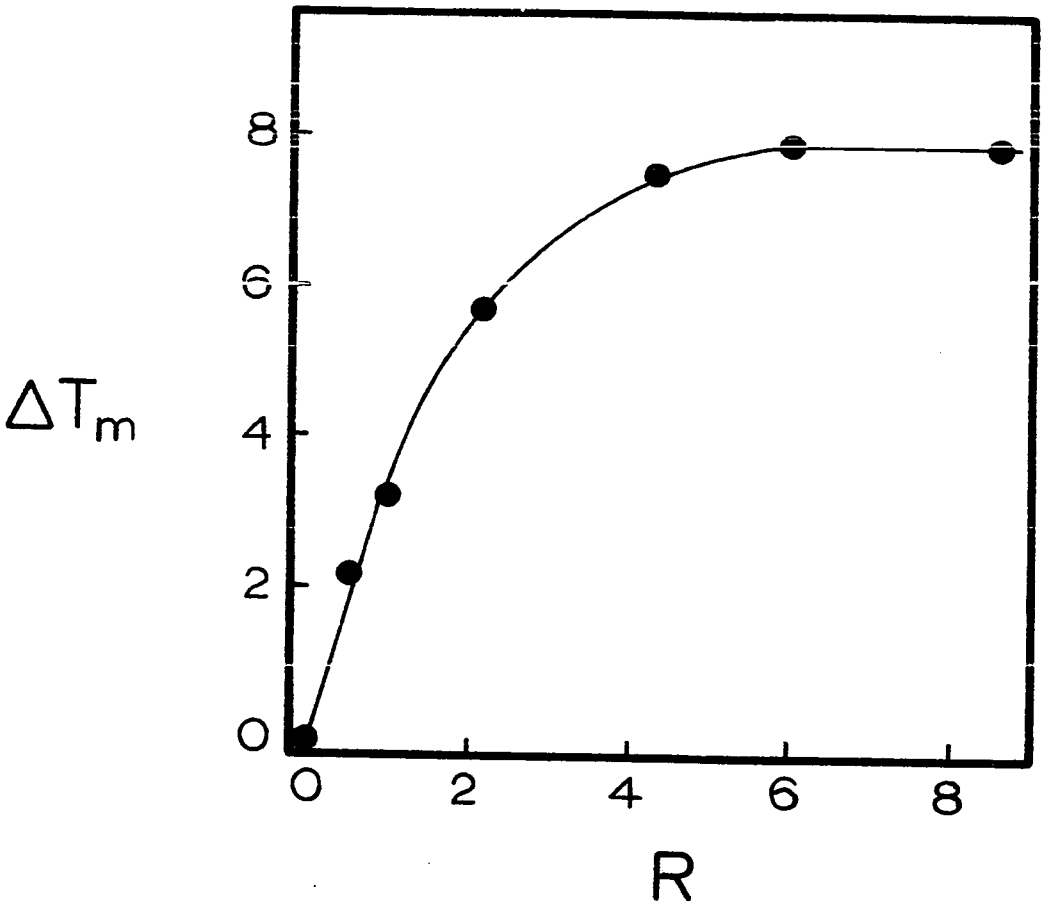
(iii) The binding of Cu(II) to RNase occurs at pH values at which most of the studies of the carboxymethylation of RNase have been carried out.

2. Thermal Denaturation

Figure 45 shows the effect of Zn(II) on the transition temperature of RNase as a plot of ΔT_m vs R. The experiments were run at R values of 0 to 8 and at pH 7.7. With reference to Figure 44, this pH value was chosen as the apparent pH of half-binding. Zn(II) does exhibit a destabilizing effect on RNase and, by comparison to Figure 25, Zn(II) is about half as effective in destabilization as is Cu(II) at equivalent values of R.

FIGURE 45

The effects of Zn(II) on the transition temperature of RNase.


ΔTm
0
2
4
6
8
0
2
4
6
8
R

V. DISCUSSION

A. Titration Curves

A key part of the analysis of any set of titration curves obtained for a protein in the presence of metal ion is the identification of the binding sites involved. In the case of the binding of Cu(II) by RNase, identification of the binding-sites has been made by Breslow and Girotti (1966) on the basis of two sets of evidence. The first of these was that the pH of proton release from the protein by Cu(II) corresponded to the expected pK_a for imidazole groups; this is shown in Figures 4 and 14. In addition, these authors measured the visible absorption spectra for RNase in the presence of Cu(II). As shown in Figure 6, the absorption maxima in the pH range 6 to 8 remain nearly constant at about 200 mμ as R is increased. This wavelength was found by Bryce and Gurd (1966) to be the absorption maximum for chelation of Cu(II) to an imidazole and two peptide nitrogens in model peptides. Evidence for chelate binding of Cu(II) is also given in Table 2, which shows that more than one proton is released for each Cu(II) bound and by Figures 4 and 14 which show that the difference titration curves do not begin to return to zero at pH values greater than pK_a for the histidyl residues.

As noted in the Historical Review, binding constants have been calculated by Saundry and Stein (1966), Breslow and Girotti (1966) and by Breslow (1967) from equilibrium dialysis, titration curves and gel filtration data, respectively. The large magnitudes of the association constants, 10^5 to 10^6 at pH 7.0, plus the absence of any detectable amounts of $Cu(OH)_2$ precipitate allows the conclusion that essentially all of the Cu(II) present in solution is bound to RNase at pH values of 7.0 or greater. Again, with reference to Figure 14, the flatness of the difference titration curves in the pH range 7 to 8 at R values of 1 to 4 allows the conclusion that binding to the sites identified by Breslow as each containing one histidyl residue is complete in this pH range. Therefore, at pH 7, R has been taken to be equivalent to $\tilde{r}$, the number of Cu(II) bound per molecule of RNase; at pH 5.5, where about a half of the proton release observed at pH 7.0 is completed, R has been taken to be $2\tilde{r}$.

A striking feature of the titration data is illustrated in Table 2 where it is shown that binding of the first three Cu(II) ions occurs with a nearly constant release of protons. Binding of the fourth Cu(II) results in some decrease in the number of protons released per ion bound. These results are explicable in terms of the large order of magnitude of the binding constant since Breslow (1967) found that, at pH 7.0, there are five sites with equivalent association constants of 3.2×10^5. At pH 5.5, Breslow (1967) assigned an association constant of 5.3×10^3 to the first binding site and a value of 5.2×10^2 to the next four.

B. Denaturation

The first conclusion that can be drawn from all of the denaturation data is that the binding of Cu(II) occurs in such a manner as to cause a destabilization of the Cu(II):RNase complex relative to RNase itself. Thus, when the complex is subjected to a denaturing medium, unfolding occurs under conditions which are milder than those needed to denature RNase. This destabilization is a general effect since it occurs both during thermal denaturation (Figure 26), and in urea solution (Figure 20).

Although Cu(II) is able to destabilize RNase, it is probable that no gross conformational change occurs in the protein as metal ion is bound. This is in contrast to the conformational change shown to accompany the binding of Cu(II) to metmyoglobin (Breslow and Gurd, 1963). If any conformational change does occur in the case of RNase, the change is not great enough to be observed by any of the techniques used in this work. The optical rotation of the complex, (Figure 21), shows no significant change from that for RNase and the magnitude of the denaturation transition, as measured spectroscopically, (Figures 18 and 20), is the same as that for RNase itself. Since this is true for either thermal denaturation or denaturation by urea, it is concluded that Cu(II) is unable to change any of the essential features of the denaturation process.

The thermodynamic parameters shown in Table 3 also support the belief that Cu(II) does not result in any gross conformational change in RNase. Since the standard enthalpy does not vary with R, the effect

of the presence of Cu(II) on the transition temperature during thermal denaturation must be interpreted as being due to the entropy of chelation of the metal ion. Qualitatively, the metal ion can be viewed as clamping the structure in the immediate area of the chelate site in a strained configuration, which would be more susceptible to unfolding that RNase itself, given an equal input of heat energy. Schrier et al (1965) find that the lowering of the transition temperature of RNase by alcohols is explainable on the basis of an entropic effect.

It is tempting then to consider that the maximum value given in Table 3 for the change in the standard entropy ($\Delta(\Delta S^o)$) determined for the thermal denaturation of RNase after the addition of Cu(II) represents the actual magnitude of the standard entropy for the reaction

$$\text{RNase} + 2\ \text{Cu(II)} \rightleftharpoons \text{Cu(II)}_2\text{RNase} \qquad (13)$$

(Two Cu(II) ions only are considered in equation 13 because the data show only two conformationally-sensitive binding sites.) However, the evaluation by Tanford (1964) and by Brandts (1964b) of the several terms which contribute to the entropy of denaturation indicates that caution should be exercised. These terms include the entropy of transfer of hydrophobic residues from within the protein fabric to the aqueous medium as well as the entropy of unfolding. Nevertheless, it is interesting that the maximum change in the standard entropy observed for RNase thermal denaturation as Cu(II) is added is of the same order of magnitude as the standard entropy values reported by Spike and Parry (1953)

for the entropy of chelation of model compounds, i.e., when four univalent nitrogenous ligands are replaced by two divalent nitrogenous ligands in the coordination sphere of divalent metal ions.

Although the reaction order plots for thermal denaturation, shown as Figures 40, 41 and 42 were initially designed to assist in the identification of the binding sites which contribute to the destabilization of RNase, the results may also be used to assist in formulating a mechanism of thermal denaturation for RNase. The simplest formulation of the denaturation process, whether or not Cu(II) is present is

$$N \rightleftharpoons D \qquad (14)$$

where all of the denatured molecules at completion of the transition are of the same type. Holcomb and Van Holde (1962) assumed the validity equation 14 as did Schrier et al (1965) in their studies of RNase thermal denaturation. Hermans and Scheraga (1961 a) calculated equilibrium constants assuming the validity equation 14 and produced van't Hoff plots of the logarithm of the equilibrium constant aginst the reciprocal of the absolute temperature. They found that the van't Hoff plots divided themselves into two sections. The portion of the curves at temperatures below the transition temperature were linear; above the transition temperature the curves show a progressively steeper slope. Since the standard enthalpy increases with an increased slope of a van't Hoff plot, Hermans and Scheraga (1961a) concluded that at the higher temperatures, different denatured forms were present and equation 14 should be written to include these additional denatured species. The results

of Holcomb and Van Holde (1962) are similar to those of Hermans and Scheraga (1961a), and it was suggested that either a sequential or a parallel production of more than one type of denatured species could be responsible for the observed curvature. Beck, Gill and Downing (1964) used direct calorimetric measurement of the thermal transition and concluded that although there was no calorimetric evidence obtained for a sequential reaction mechanism, parallel production of different denatured states was not ruled out. Scott and Scheraga (1963) employed a stopped-flow spectrophotometric technique to measure the kinetics of thermal denaturation of RNase. These authors found that the kinetics were complex but separable into two parallel reversible reactions, each involving a different tyrosyl residue. These residues were concluded to be the A and B tyrosyls of Bigelow (1961). Not all of the experiments listed above were carried out at the same pH values but this probably does not matter since the data of Hermans and Scheraga (1961a) show parallel van't Hoff plots over the pH range of 2 to 7.

Scott and Scheraga (1963) also made equilibrium measurements of the thermal denaturation process and were able to resolve their data into two separate parallel denaturation processes which could be summed to produce the observed experimental values of $\Delta\varepsilon$ vs temperature. The following mechanism was then offered:

$$N \rightleftharpoons D_1$$

$$N \rightleftharpoons D_2 \qquad (15)$$

This mechanism assumes parallel denaturation reactions, where two separate regions of the RNase molecule denature essentially independently, each at its own rate and each possessing its own thermodynamic parameters. If equation 15 is the correct interpretation of the denaturation process, then denatured state I of Bigelow (1964) could be identical to D_1 in equation 15 and denatured state II could be observed as the summation of D_1 and D_2.

The reaction order data are interpretable in terms of a mechanism for denaturation which is at least similar to equation 15. According to these data, destabilization by Cu(II) at pH 5.5 is a result of the binding of one metal ion. A second Cu(II) is seen to assist in destabilization at about the value of log K at which the various van't Hoff plots discussed above show the change to higher values of the standard enthalpy. Apparently, the denaturation step which normalized tyrosyl B is facilitated by the binding of one Cu(II) ion. The denaturation process which produces the observed state II is then assisted by the binding of the one Cu(II) ion which participated in the first step as well as by a second ion. One Cu(II) ion must participate in both steps since there is no evidence that a total of more than two Cu(II) ions bind at conformationally-sensitive sites in the pH range studied. Although the effects of Cu(II) are discussed here in terms of the mechanism of equation 15, it is not implied that the reaction order data support that particular mechanism. The mechanism provides a convenient example for discussion; what is

important is that the reaction order provide one of the few known sets of experimental evidence for a two-step mechanism for thermal denaturation of RNase.

The effect of pH on the Cu(II) destabilization of RNase is shown in Figures 26, 27, and 28. Not only is Cu(II) less effective in the lowering of the transition temperature at pH 7.0 than at pH 5.5, but the pH 7.0 data shows an anomaly in the shape of the curve in all three Figures. There are at least two possible explanations for the variation with pH of the extent of destabilization by Cu(II). These are:

(i) A change in the number and identity of the groups bound to the metal ion.

(ii) A change in the local environment around the binding site as a result of ionization of titratable groups in the binding-site.

Either of these could happen and either might cause changes in the relative values of the equilibrium constants for the different sites such that binding might change from random to sequential or even cooperative. But from the work of Girotti and Breslow (1966) it is unlikely that the number and identity of the ligands coordinated to Cu(II) are different at pH 7.0 than they are at pH 5.5; at either pH the first four sites appear to consist of an imidazole group and two adjacent peptide nitrogens. Therefore the ionization of titratable groups, i.e., the four histidyl residues, must result in a change in the conformation of the protein in the regions adjacent to and including the Cu(II) binding sites. However, this change is not extensive enough to affect significantly the transition temperature of RNase over this pH range (Figure 25) in the absence of

Cu(II). It is interesting that Cathou and Hammes (1964, 1965) and French and Hammes (1965) have used temperature jump studies to show that RNase undergoes a pH dependent isomerization with a pK value corresponding to the protonation of one or more imidazole groups. (French and Hammes (1965) also cite unpublished evidence from Tanford and Brink for a very slight variation of optical rotatory power for RNase with pH; the pK associated with this change is about 6.0.) The isomerization is said to involve the formation of a hydrogen bond between an ionized carboxyl group and a protonated imidazole group, and such an isomerization could explain the different effects of Cu(II) on the transition temperature at pH 5.5 compared to pH 7.0. This would be particularly true at low values of R, where one or more histidyl residues would be free to hydrogen bond without interference from Cu(II). It is concluded that a small conformational change produces the difference between the shapes of the two curves, at pH 5.5 and 7.0, in Figure 28. These data allow the important conclusion that saturation of the binding sites proceeds in a sequential manner at pH 7.0 as opposed to a more nearly random saturation at pH 5.5. The random saturation at pH 5.5 is deduced from the smooth curve presented by the data for the variation of the change in the transition temperature with R, whereas the abrupt changes in the slope of the pH 7.0 curve are difficult to explain unless sequential binding as assumed.

It is now possible to evaluate further the suggestion that cooperative effects are present when Cu(II) is bound to RNase (Girotti

and Breslow, 1966). The data presented in Figure 24 for the denaturation in the presence of Cu(II) at pH 5.5 produce a featureless plot and show no evidence of cooperative effects. This conclusion is not contradicted by the data in Figure 34, showing the results of Cu(II) inhibition of carboxymethylation of RNase at pH 5.5. If cooperative effects are present at all, they are most likely to be taking place at pH 7.0, as evidenced by Figure 28. It seems probable, as shown above, that the sequential binding observed is caused by small conformational changes as each site is occupied, which results in the first four binding constants not being equivalent. Non-equivalent binding constants could account for the sequential binding observed. It is true that the findings of Breslow (1967) are consistent with equivalent binding constants but it seems unlikely that all of the first five sites should have strictly identical constants. The data in Figure 28 clearly distinguishes two conformationally-sensitive binding sites with different values for their association constants or cooperative binding.

It is then concluded that no conclusive evidence for cooperative binding is apparent in any of the denaturation data. At pH 7.0, the first and fourth Cu(II) ions are bound at conformationally-sensitive sites. At pH 5.5, there are also two conformationally sensitive binding sites. It is assumed that the identity of these sites does not change with pH.

C. Kinetics of Formation of Carboxymethylated Ribonucleases

Figure 34 shows the effects of Cu(II) in inhibiting the carboxymethylation of RNase at pH 5.5. The binding of one Cu(II) ion completely

inhibits the reaction. Since Figure 30 and the work of Crestfield et al (1963 c) show that histidyl 119 is the predominant product of the reaction at pH 5.5, it is evident that the inhibition is effected at a Cu(II) binding site containing the histidyl 119 residue. Although this conclusion had been reached previously in the work of Crestfield et al (1963 c) and Heinrikson (1966), Figure 33 allows the additional conclusion that the first Cu(II) ion bound at pH 5.5 is bound at a site containing histidyl 119. This conclusion is of importance in the assignment of individual binding sites to specific locations in the sequence of amino acid residues in RNase.

The results of the experiments measuring the inhibition of carboxymethylation of RNase at pH 7.0, shown in Figure 34, are again seen to be both qualitatively and quantitatively different from the results at pH 5.5 (Figure 33). In this case, however, the differences are more easily interpreted than they were in the case of thermal denaturation. The kinetic constants for the reaction in the absence of Cu(II) may be used to calculate the times at which one-half and one-fourth of the initial concentration of RNase has reacted, at pH 5.5 and 7.0 respectively. The equations used are:

$$t\,1/2 = (2.303 \log 2)/k \qquad (16)$$

and

$$t\,1/4 = (2.303 \log 1.33)/k \qquad (17)$$

Equations 16 and 17, plus the pH-stat recordings used in the calculations of Figures 31 and 32, may be used to show that, at either pH,

the carboxymethylation reaction would be complete when one proton per RNase molecule has been released, in spite of the fact that more than one residue is reacting. This important finding has not been previously reported for the reaction at pH 7.0 although it has been emphasized by Crestfield et al (1963 c) for the reaction at pH 5.5. They explained the restriction of the extent of reaction as being due to the orientation of the attacking iodoacetate anion by the postively-charged histidyl 12 residue. Supporting evidence for their view consists of the much slower rate of attack by the uncharged iodoacetamide (Stark, Stein and Moore, 1959). Although this orientation effect can explain the difference between the rate constants observed in the absence of Cu(II) at pH 5.5 and 7.0 (Figures 31 and 32), it should not be taken as the explanation for the confinement of the reaction to a total of one residue per protein molecule. This is probably true at pH 5.5 and is certainly true at pH 7.0 where both the histidyl 119 and the histidyl 12 residues are nearly completely unprotonated; hence neither could orient an attacking anion.

The results shown in Figures 33 and 34 are then best interpreted as follows. At pH 5.5, the expected alkylation at the histidyl residues at positions 119 or 12 occurs (Figure 30). At pH 7.0, carboxymethylation occurs at these two histidyl residues plus the lysyl 41 residue (Gundlach et al, 1959; Glick et al, 1966). Methionyl residues may also react at pH 7.0, but this reaction does not result in the net release of protons (Glick et al, 1966) and hence does not contribute to

the reaction rate as determined by the pH-stat; further, if methionyl residues do react at pH 7.0 in the alkylation by iodoacetate, they do so with an extremely slow reaction rate (Heinrikson, 1966). All three of the histidyl residues at positions 12, 119 and 48 and the lysyl residue at position 41, are located at or near the active center of RNase, i.e., within or near the crevice illustrated in Figure 2. Since a total of only one residue is alkylated at either pH 5.5 or 7.0, it is concluded that only one bulky carboxymethyl group can be fitted within this crevice; where the carboxymethyl group is attached depends both on steric considerations and on any electrostatic orientation effects operative at the pH of the reaction. When Cu(II) is present, additional restrictions are imposed upon the location of the carboxymethyl group depending on the number of Cu(II) ions bound, and where they bind.

In summary, the binding of the first Cu(II) at pH 5.5 to a site containing histidyl 119, known to be the residue at which about 90% of the reaction occurs, produces almost complete inhibition of carboxymethylation (Figure 33). This Cu(II) is bound about one order of magnitude more strongly than the succeeding three Cu(II) ions (Breslow, 1967). At pH 7.0 the inhibition results (Figure 34) suggest that random binding of Cu(II) occurs at the residues being carboxymethylated, in contrast to the sequential binding indicated by the thermal denaturation results (Figure 28). However, the denaturation experiments reflect only the binding of Cu(II) at the two conformationally-

sensitive sites whereas the kinetic results at pH 7.0 reflect the binding of at least three Cu(II) ions at histidyls 119, 12 and 48, and since the inhibition observed at pH 7.0 has been shown to be the result of both the binding of Cu(II) and steric hindrance, there is no reason to expect that the data should reflect sequential binding.

D. Tentative Assignment of Conformationally-Sensitive Binding Sites

Of the four histidyl-containing binding sites, two have been shown to be conformationally-sensitive and two have no effect on the stability of RNase. The first Cu(II) ion bound at either pH 5.5 or 7.0 appears to bind at a conformationally-sensitive site containing histidyl 119. The evidence for this view is abundant, both from this work and from the work of authors cited in the previous section. Since Figure 33 shows that the first Cu(II) is bound at pH 5.5 to histidyl 119 with nearly complete inhibition of the carboxymethylation reaction at that residue, the corollary may be drawn that the enzymic activity should also be nearly destroyed when the first Cu(II) ion is bound. It is observed (Gundlach et al, 1959) that the isolated product, 1-carboxymethylhistidyl-119-ribonuclease, has no enzymic activity. However, the assay data of Ross et al (1962) and Davis and Allen (1955) do not show total inhibition of enzymic activity until values of R as great as 10^3 are reached. Similar results were obtained in the recent work of Takahashi, Irie and Ukita (1967). Although these seemingly contradictory results can be partially explained as the result of competition

by Cu(II) and substrate for the same binding sites on the protein (Barnard and Stein, 1959; Ross et al, 1962; Breslow and Girotti, 1966), nucleotide substrate is undoubtedly also competing with the enzyme for Cu(II). With reference to Figure 3, RNase substrates contain nitrogenous bases which could be expected to bind Cu(II) over the pH range of assay. Wacker and Vallee (1959), Wacker (1962), and Wojnar and Roth (1963) have demonstrated that firm binding does occur.

Before attempting an assignment of the second conformationally-sensitive site, the model of RNase, deduced by Harker et al (1967) from their x-ray crystallography data, may be examined further. Figure 2 shows that three of the histidyl residues are located in or near a crevice. Part or all of this crevice is the active center of RNase. The three histidyls at or near the active center are residues 12, 119 and 48. Residues 12 and 119 lie within the crevice and, as mentioned previously, are essential for enzymic activity, but there is no evidence that residue 48 is essential for activity. Histidyl 105 is some distance from the crevice illustrated in Figure 2 and its side chain appears to be oriented towards the medium. For this reason, and from the denaturation evidence which follows, it seems logical to eliminate histidyl 105 from further discussion, and the second conformationally-sensitive site must include either histidyl 12 or 48.

Additional evidence for considering histidyl 12 or 48 to be in a

conformationally-sensitive region of the protein molecule is available from the literature. It is consistently observed that acidic media are able to unfold a portion of the RNase molecule to produce either denaturation state I or II (Bigelow, 1964), with tyrosyl B or both B and A, respectively, normalized. It has been shown by Woody, Friedman and Scheraga (1966) and Li, Riehm and Scheraga (1966) that tyrosyl B occupies position 92 and tyrosyl A occupies position 25 in the amino acid sequence of RNase. Tyrosyl B only may be normalized at low pH and low temperatures (Bigelow, 1961) and is situated in a loop (Figure 2) which can be visualized (Bigelow, 1967) as becoming loose under mild denaturing conditions. Tyrosyl A can be seen (Figure 2) to be located near the first disulfide bond from the amino terminus. It is reasonable to believe (Bigelow, 1967) that denaturation state II is obtained when the loop containing tyrosyl B is loosened from the surrounding structure and when a disruption occurs in the secondary bonding of the amino terminal segment to the rest of the molecule. Conditions which are known to disrupt this secondary bonding to normalize tyrosyl A include heat, acid and some organic solvents. These conditions are summarized by Bigelow (1964), and are more severe than those which normalize tyrosyl B only. It is important to note that, even though the normalization of these two tyrosyl residues may be causally related, denaturation states I and II can occur as essentially independent processes. Thus the known features of the

RNase molecule are consistent with the evidence for a two-step denaturation mechanism indicated by the reaction order data in Figures 40, 41 and 42.

Other lines of evidence support the belief that heat and acidic media can rupture the forces holding the amino terminal end to the remainder of the RNase molecule. Crestfield et al (1962) showed that active dimers of RNase could be formed when the enzyme is lyophilized from 50% acetic acid solution. In these dimers, the amino terminal peptide of one RNase molecule combines with the remainder of another to produce the conformation necessary for activity. Richards (1955) has reacted RNase with subtilisin, a protease from B. subtilis. Cleavage occurred quickly only at the peptide bond between residues 20 and 21 leaving two fragments (which Richards called RNase-S-peptide for the peptide containing residues 1-20, and RNase-S-Protein for the longer fragment of residues 21-124); the fragments remain associated under mild conditions. Richards and Vithayathil (1959) found that these fragments could be separated in acid media or at elevated temperatures showing that the secondary forces holding them together could be ruptured under the same conditions that can normalize tyrosyl A in denaturation. Potts and Sherwood (1965) showed that tyrosyl A is normalized when RNase-S-peptide is separated from RNase-S-protein.

The importance of the histidyl residues to all of these processes

may be seen with reference to Figure 2, and to stereoscopic slides of the structure of the associated RNase S which were available in this laboratory through the kindness of Dr. H. W. Wyckoff (Wyckoff, Richards, Doscher, Tsernoglou, Inagami, Johnson, Hardman and Allewell, 1967). Histidyl residues 119 and 48 are located in the two regions of the RNase molecule which could be most effective in the binding of the amino terminal segment. Histidyl 12 is, of course, in this segment, and is spatially adjacent to the histidyl at position 119. Therefore, the binding of Cu(II) at any of these three residues could be expected to assist in the formation of denatured state II through the rupture of the forces binding the amino terminal segment to the rest of the molecule.

It is more difficult to decide whether histidyl 12 or 48 is part of the second conformationally-sensitive binding site for Cu(II). However, Breslow and Girotti (1966) have shown that the binding of two equivalents of either 2'-cytidine monophosphate or 3'-cytidine monophosphate can modify the binding of at least the first two Cu(II) ions at pH 5.5. It is shown in Figure 24 that the first two Cu(II) ions bind at pH 5.5 to the two conformationally-sensitive sites. Therefore, it follows that histidyl 12 is at the second conformationally-sensitive site because it is difficult to believe that a bulky nucleotide would bind at histidyl 48 rather than at the more sterically available histidyl 12. Various authors, including Nelson, Hummel, Swenson and

Friedman (1962), Crestfield et al (1963 c) and French and Hammes (1965) consider histidyl 12 to be intimately involved in the binding of nucleotide. Further, the steroscopic slides of Wyckoff et al (1967) show that histidyl 48 is nearly buried by the terminal peptide. It may then be considered to be inaccessible to nucleotide, although not to Cu(II). The second conformationally-sensitive site is then tentatively assigned as the one which contains histidyl 12.

The reason for the reversal in the order of binding of Cu(II) as the pH is varied from 5.5 to 7.0 must be found in the small conformational change in RNase believed to occur as a function of pH. (Hammes and French, 1965). If histidyl 12 is at the second conformationally-sensitive site, then the second Cu(II) ion, at pH 5.5, and the fourth Cu(II) ion, at pH 7.0, are bound to this residue (Figure 28). A possible explanation for this reversal can be obtained from the work of Hammes and French (1965) on the isomerization of RNase. If residue 48 is the histidyl involved in hydrogen bond formation to a carboxylate residue, then, at pH 5.5, the binding of Cu(II) to it might be sufficiently retarded to reverse the sequence of Cu(II) binding observed at pH 7.0. At pH 7.0, this residue would be ionized and could not engage in the type of hydrogen bonding postulated by Hammes and French (1965). It is true that these authors concluded that histidyl 119 was the residue involved in hydrogen bond formation but the spatial location of the histidyl residue at position 48 was not known at

the time of their work and definite assignments of pK_a values for the various histidyl residues are not yet available. Therefore, even though the explanation above is conjectural, it must remain as an interesting possibility.

Thus, from this work and the work of many others, it would appear that histidyl residues 119 and 12, and possibly histidyl 48, have roles in maintaining the native structure of RNase, whereas residue 105 does not.

E. Experiments with Zn(II)

It was noted in the Historical Review that there is a basic difficulty in the interpretation of the titration curves for RNase in the presence of Zn(II) (Figures 5 and 44). The fact that the difference titration curves do not return to zero in the alkaline pH region may be taken as evidence that a chelate binding site is involved, but this is not conclusive proof since it seems possible that hydrolysis of the metal ion could result in the release of one of the protons per Zn(II) titrated in the difference curve. If only one proton released is due to hydrolysis, i.e.,

$$Zn(H_2O)^{++} \rightleftharpoons Zn(OH)^{+} + H_3O^{+} \qquad (18)$$

the metal ion could remain bound to the protein and precipitation of $Zn(OH)_2$ would not be observed.

Both Ross et al (1962) and Breslow and Girotti (1966) favor the

interpretation that one or more histidyl residues at the active center of RNase are involved in the binding of Zn(II). Their interpretation is supported by the known inhibition of RNase activity by Zn(II) and by the modification of nucleotide binding when Zn(II) is present as reported by either of these authors. But it is this interpretation which forced Breslow and Girotti (1966) to divide their difference titration curves (Figure 5) into two sections, one on either side of pH 7.0. Each section of the curve was said to result from a different type of binding since proton release from histidyl groups by metal ions can only occur in or below the normal pH region of titration for these groups.

The difference titration curves in the presence of Zn(II) could also be viewed as arising from the binding of the metal ion to several chelate sites involving lysyl groups. This interpretation would not demand the division of a nearly symmetrical difference titration curve into two section, but it would demand that the inhibition of RNase activity and the effects of nucleotide binding originate from the binding of Zn(II) at the lysyl 41 position. Again, lysyl 41 is essential for enzymic activity (Hirs, 1962; Hirs and Kycia, 1965) and is near the active center (Figure 2). Another alternative for interpretation of the titration results would be the separation of the difference curve into two sections corresponding to binding of the metal at both histidyl and lysyl residues.

In the absence of a satisfactory interpretation of the titration data, the results of the thermal denaturation experiments in the presence of Zn(II), shown in Figure 45, are also subject to uncertainties. The most important of these is that it is not possible to judge the extent of binding of the metal ion to the protein. If it is true that half of the Zn(II) added is bound at pH 7.7, Zn(II) is as effective in destabilization as is Cu(II). But, if the binding of Zn(II) to conformationally-sensitive sites containing histidyl residues is complete at pH 7.0, then Zn(II) is about half as effective as Cu(II). The reaction order for destabilization by Zn(II) could then be either 1 or 2.

Experiments designed to answer some of these questions are being planned in this laboratory.

VI. SUMMARY

The binding of Cu(II) to RNase at pH values of 7.0 and lower occurs most strongly at chelate sites involving the four histidyl residues known to be present in RNase. The titration studies carried out in this work agree well with those of Breslow and Girotti (1966) who showed that, in addition to one of the histidyl residues, two adjacent peptide nitrogens are coordinated to each Cu(II) ion.

As Cu(II) ion is bound to RNase, the stability of the protein, both in urea solution and at elevated temperatures, is decreased. It is shown by the variation in the transition temperature for RNase in the presence of Cu(II) that only two of the four histidyl residues are located in conformationally-sensitive regions of the molecule. The reaction order equations derived support this conclusion and, in addition, plots of these equations show that the thermal denaturation of RNase occurs by a two-step mechanism.

The order in which the four histidyl-containing binding sites are filled by Cu(II) varies as the pH is increased. From pH 4.5 to pH 5.5 the first two Cu(II) ions are bound at the two conformationally-sensitive sites. Above pH 5.5 the order of binding begins to vary; at

pH 7.0 the first and fourth Cu(II) ions are bound at the conformationally-sensitive sites whereas the binding of the second and third ions does not affect the stability of RNase.

Cu(II) is also shown to inhibit the carboxymethylation of the histidyl residues at low molar ratios of metal ion added to protein. At pH 5.5, the binding of one Cu(II) ion almost completely inhibits the alkylation of the histidyl at position 119; this residue forms about 90% of the product of the reaction at pH 5.5. The binding of larger amounts of Cu(II) completes the inhibition and carboxymethylation is no longer observed at this pH. At pH 7.0, four Cu(II) ions can inhibit carboxymethylation, although not completely. The extent of inhibition increases as each Cu(II) ion is bound. It is shown that a total of only one residue per RNase molecule can react at either pH and that the observed inhibition by Cu(II) at pH 7.0 is predictable from steric considerations and should not be interpreted as reflecting the order in which the binding sites for Cu(II) are saturated.

The assignment of one of the two conformationally-sensitive binding sites for Cu(II) as the region of the molecule which contains the histidyl 119 residue is definite. This site is the first to bind Cu(II) over the whole pH range studied. The second conformationally-sensitive site could include either of histidyl 12 or 48. Histidyl 105 is eliminated from consideration because it is isolated from those regions of the protein molecule which are believed to unfold during

the denaturation process. Histidyls 119 and 12 have been shown by a number of workers to be essential for enzymic activity, but the three-dimensional models of the molecule deduced by Harker et al (1967) and by Wyckoff et al (1967) from their x-ray crystallography studies shows that all three of the histidyls 119, 12 and 48 are located within or near the crevice which contains the active center of the enzyme. Histidyl 12 is, however, tentatively preferred over histidyl 48 in the assignment of the second conformationally-sensitive binding site for Cu(II) because of steric considerations. Histidyl 12 is more ideally situated for the binding of nucleotide substrate and Breslow and Girotti (1966) show that competitive inhibitors can modify the binding of the first two Cu(II) ions at pH 5.5. Since, at this pH, the first two Cu(II) ions bound are shown to be located at the two conformationally-sensitive sites, the histidyl 12 residue is believed to be contained in one of these sites.

The suggestion is offered that the histidyl residue at position 48 is involved in a small conformational change which is responsible for the variation with pH of the order in which the four Cu(II) binding sites are filled. French and Hammes (1965) have shown that RNase isomerizes over the pH range of histidyl ionization accompanied by the formation of a hydrogen bond between a protonated histidyl and an ionized carboxyl group. If histidyl 48 is involved in hydrogen bonding, the binding of Cu(II) to it could be retarded at pH 5.5. At pH 7.0, this

residue would be ionized and could bind Cu(II) according to its intrinsic association constant.

Some preliminary studies were also carried out on the binding of Zn(II) to RNase. The interaction was studied by titration curves produced in the presence of Zn(II), and by thermal denaturation experiments conducted at pH 7.7. This is the apparent pH at which about half of the Zn(II) added to the solution is bound to the protein. It is shown that Zn(II) is able to lower the transition temperature of RNase, but several problems in the interpretation of the titration curve make it difficult to draw definite conclusions from the denaturation data. The most important of these problems is that the titration data do not allow a knowledge of the number and identity of the ligands from the protein molecule which coordinated to the Zn(II).

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APPENDIX I

A COMPARISON OF THE THERMAL DENATURATION PROPERTIES OF SIGMA AND MANN RIBONUCLEASES A

Since the RNase A preparation purchased from the Sigma Chemical Co. showed less stability to storage in the dry frozen state than did a similar preparation purchased from the Mann Research Laboratories, it was necessary to determine if there were any observable differences in the thermal denaturation behavior of the two protein samples. Therefore, certain key denaturation experiments were repeated using the Mann lot of RNase A.

Table 4 shows the results of thermal denaturation of the Mann RNase A at an R value of 2.06 at pH 5.5 and 7.0, and at an R value of 4.12 at pH 7.0. These results are compared in Table 4 with those obtained using the Sigma sample (Figure 27). It can be seen that the two lots give very nearly identical results and it is concluded that the heterogeneity present in the Sigma sample was insignificant in its effect on thermal denaturation and metal-binding properties. Table 4 also is of importance in that it provides conformation of some critical values of the transition temperature.

TABLE 4

A COMPARISON OF THE THERMAL DENATURATION BEHAVIOR OF THE MANN AND SIGMA LOTS OF RNASE A IN THE PRESENCE OF CU(II). VALUES PRESENTED ARE OF ΔT_m.

Source	R	pH	ΔT_m
Mann	2.06	5.5	8.6
	2.06	7.0	8.1
	4.12	7.0	16.8
Sigma	2.15	5.5	9.1
	2.15	7.0	8.4
	4.24	7.0	17.9

APPENDIX 2

THERMAL DENATURATION OF 1-CARBOXYMETHYL-HISTIDYL-119-RIBONUCLEASES

This derivative was obtained from the Mann Research Laboratories and its thermal denaturation properties were measured in the presence and absence of Cu(II) at pH values of 5.5 and 7.0 in 0.1 M KCl. In contrast to RNase, the derivative tended to aggregate in its denatured form at pH 7.0 and with Cu(II) at pH 5.5 when R was greater than 2. For this reason, log wavelength vs log absorbance plots (Wetlaufer, 1962; Bigelow, 1961) were used to correct the difference spectra for light scattering at pH 7.0 in the absence of metal ion. Calculations to determine values of $\Delta\varepsilon$ in the presence of Cu(II) were carried out as described for RNase and illustrated in Figure 17.

Figure 46 shows the results of thermal denaturation of the derivative at pH 5.5 and R values of 0.0, 0.89 and 1.78; as well as results at pH 7.0 and R values of 0.0 and 0.89. The curve in Figure 46 was drawn through the points obtained at pH 5.5 in the absence of Cu(II) since there was no aggregate formation under these conditions and the experimental points provide a smoother curve and, by inference, greater accuracy. It can be seen that the transition temperature

remains constant, within experimental error, in all of the experiments reported. Thus, neither the protonation of the unmodified histidyl residues nor the presence of Cu(II) has any effect on the stability of this derivative. It is also seen that the magnitude of the denaturation transition, $\Delta\mathcal{E}$, for the 1-carboxymethylhistidyl-119-ribonuclease is significantly lower than that for RNase. Figure 46 shows a total change of 1170 $\Delta\mathcal{E}$ units for the derivative, a value which is intermediate between the 700 units observed for denatured state I and the 1700 units observed for denatured state II of RNase (Bigelow, 1964). All of these results taken together indicate that carboxymethylation of the histidyl residue 119 at the active center of RNase produces a derivative which has one of the three anomalous tyrosyl residues in RNase partially exposed to the solvent medium. In addition, the reaction order for destabilization of the derivative is necessarily zero and it would appear that abolishment of a Cu(II) binding site involving histidyl 119 removes the site which is able to initiate destabilization of RNase, a conclusion which is consistent with the results of all of the work presented in this thesis. Further, the derivative has a much lower transition temperature than RNase itself, supporting the idea that either carboxymethylation or the binding of Cu(II) at histidyl 119 produces destabilization of RNase.

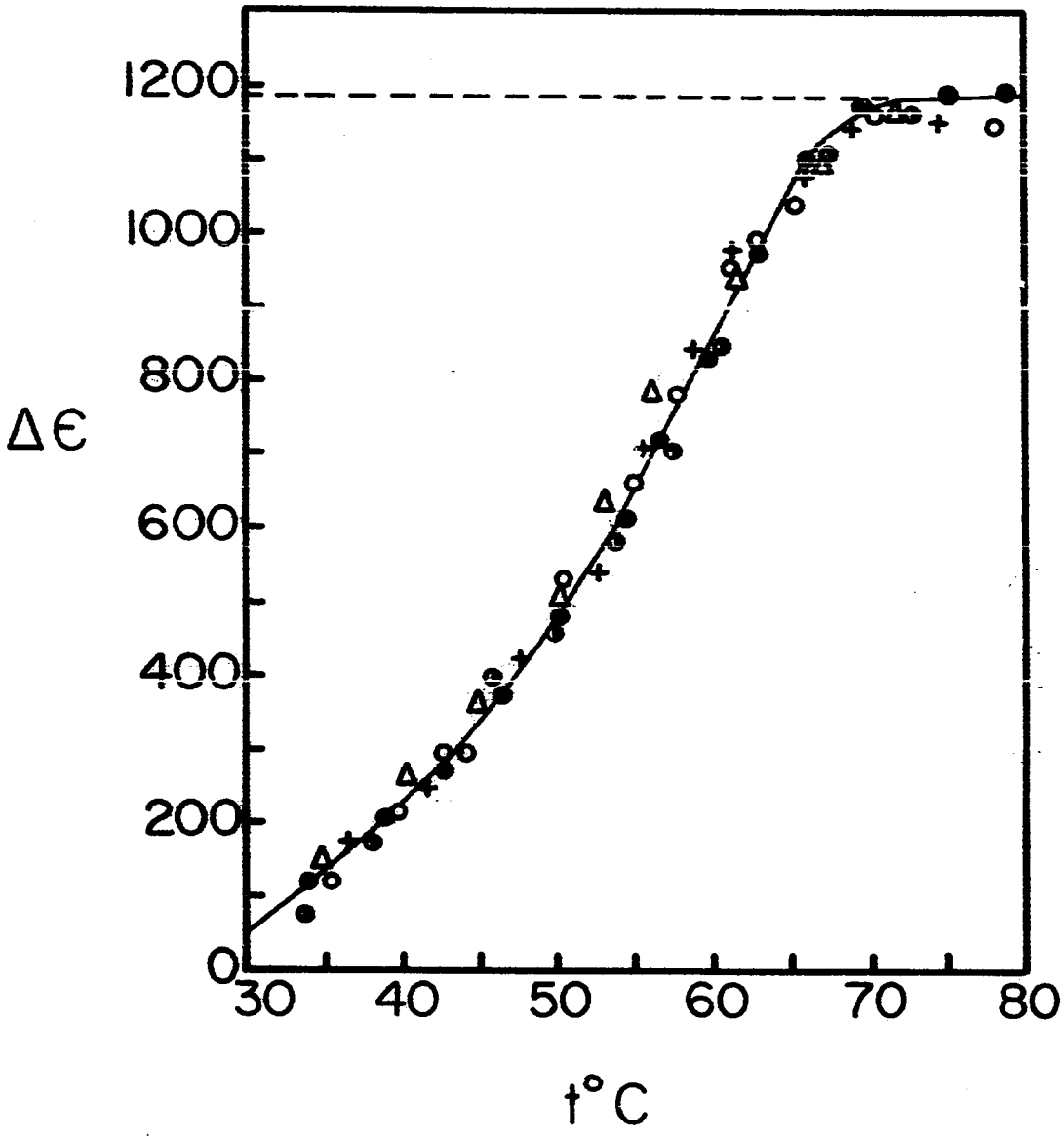
These results are self-consistent throughout but a difficulty arises in that Yang and Hummel (1964) observed the thermal denaturation

FIGURE 46

Thermal denaturation of 1-carboxymethylhistidyl-119-ribonuclease in 0.1 M KCl in the presence and absence of Cu(II).

pH 7.0: O O, R=I; + +, R=0.89.

pH 5.5: ● ●, R=0; ◑ ◑, R=0.89; ▲▲, R%1.78.


1200
1000
800
600
400
200
0
Δε
30
40
50
60
70
80
t° C

of 1-carboxymethylhistidyl-119-ribonuclease and found that the derivative had essentially the same transition magnitude, but a slightly higher transition temperature than does RNase itself. The results of Yang and Hummel (1964) and the results from this work are summarized together in Table 5. If both sets of data are accepted as correct, the most reasonable explanation for the discrepancy would seem to be a difference in the protein sample used by these authors and the sample used here. The sample used in this work was the ethanol precipitate of 1-carboxymethylhistidyl-119-ribonuclease prepared by Mann Research Laboratories. Since the ethanol precipitation is not a part of the procedure outlined by Crestfield et al (1963b) and followed by Yang and Hummel (1964), it is possible that the treatment could have some effect on the structure of the protein. Treatment with ethanol could be considered to be a mild one and it is possible that some loosening of the portion of the molecule containing tyrosyl B takes place (Bigelow, 1967).

Gally and Edelman (1964) found, in a series of thermal denaturation experiments with 1-carboxymethylhistidyl-119-ribonuclease using fluorescence techniques, that the transition temperature for the derivative is not a function of pH over the range of pH 4.5 to 9.0. This finding partially supports the results shown in Figure 46, although Gally and Edelman (1964) support the findings of Yang and Hummel (1964) in reporting the actual values of the transition temperature to

TABLE 5

COMPARATIVE VALUES OF SOME THERMAL DENATURATION PARAMETERS FOR 1-CARBOXYMETHYLHISTIDYL-119-RIBONUCLEASE

Source	pH	T_m	Transition Magnitude in Units
This work	5.5	54.0°	-1170
	7.0	54.0°	-1170
Yang and Hummel	5.0	62.8°	-1860
	8.0	60.5°	-1870

be somewhat higher than those for RNase.

It is believed that Cu(II) does not affect the transition temperature of this carboxymethylated derivative, but the thermal denaturation properties of 1-carboxymethylhistidyl-119-ribonuclease itself clearly require additional investigation before further attempts at measurement in the presence of metal ions are carried out. Such investigations are being planned in this laboratory.

APPENDIX 3

DERIVATION OF EQUATIONS DESCRIBING THE EFFECT OF CU(II) ON THE THERMAL DENATURATION BEHAVIOR OF RIBONUCLEASE

A. Determination of the reaction order for destabilization of RNase by Cu(II)

Barnard (1964 a) and Tanford and De (1961), measuring urea denaturation of RNase and β-lactoglobulin respectively, produced plots of the logarithm of the equilibrium constant vs the logarithm of the concentration of urea where the slope was said to be n, the number of molecules of urea interacting with the protein to cause denaturation. There are numerous other examples of similar plots in the literature.

In the examples cited, K is the equilibrium constant where it is assumed that the equilibrium may be described as

$$N \underset{}{\overset{K}{\rightleftharpoons}} D \qquad (14)$$

Generally, values of K which appear in the plots are taken only from the region of transition. N and D refer to native and denatured protein molecules in a one-step transition. The approach is similar to kinetic determinations of the reaction order, which is assumed to be

a measure of the molecularity of the reaction. Plots of kinetic data to determine the reaction order will of course vary in form according to the mechanism of the reaction studied. The denaturation of RNase by urea was shown by Barnard (1964 a) to be first-order kinetically with respect to protein. His equation for the reaction order with respect to urea is

$$n = \frac{d \log k}{d \log (\text{urea})} \qquad (19)$$

where k is the first order rate constant for the disappearance of native protein or appearance of denatured protein. Barnard used the same equation for his equilibrium data, with K substituted for k. In both instances plots of the logarithm of the constant against the logarithm of the urea concentration were linear and both were said to give a slope, n, equal to the number of urea molecules taking part in the reaction. Alternatively, n may be called the reaction order.

Substituting the equilibrium constant and integrating, equation 19 may be written as

$$\log K = n \log (\text{urea}) + B \qquad (20)$$

Or, in terms of activities:

$$\log K = n \log \gamma (\text{urea}) + B \qquad (21)$$

This equation justifies the linear plot observed and indicates that the integration constant B is equal to log K when the urea activity is unity.

The number of urea molecules taking part in denaturation, 16 in Barnard's (1964 a) study, was negligible compared to the total urea concentration and does not affect the validity of equation 21, i.e., essentially all of the urea is free urea.

According to the definition of B given above, equation 21 may also be written as

$$\log K = n \log \gamma\,(\text{urea}) + \log K' \qquad (22)$$

and an analogous equation is written for the observed effects of Cu(II) on the thermal denaturation of RNase. This equation is

$$\log K = n \log \gamma\,[\text{Cu(II)}] + \log K'' \qquad (23)$$

where K'' is the equilibrium constant in the presence of Cu(II) at unit activity. Equation 23 may be rearranged to

$$\log K = \log K'' + n \log \gamma + n \log [\text{Cu(II)}] \qquad (24)$$

and to

$$\log K = Q + n \log [\text{Cu(II)}] \qquad (25)$$

where

$$Q = \log K'' + n \log \gamma \qquad (26)$$

and has no measurable physical significance.

In contrast to the situation with urea denaturation of RNase, much of the Cu(II) in solution is bound to protein. The derivation to this point has dealt only with free Cu(II) and is therefore not particularly useful unless values of γ are available. But since Table 2

shows that $\bar{r}$ is nearly independent of R so long as only the histidyl-containing binding sites are being considered, use may be made of the following relation:

$$[Cu(II)] = R\,[RNase]\,(1 - \bar{r}) \tag{27}$$

where [RNase] is the total protein concentration. If equation 27 is substituted into equation 25:

$$\log K = Q + n \log \{R\,[RNase]\,(1 - \bar{r})\} \tag{28}$$

which may be arranged to give:

$$\log K = Q + n \log R + n \log \{[RNase]\,(1 - \bar{r})\} \tag{10}$$

Thus a plot of log K vs log R should give a linear slope equal to the reaction order, n, which is taken to be a measure of the number of Cu(II) ions contributing to destabilization. It is important to note that equation 10 may be used only when metal ions are binding to any given sites in a random fashion. In addition, equation 10 may not be considered to be a general form, since the near-constancy of $\bar{r}$ with R is an unusual property of RNase.

B. Determination of the enthalpy of thermal denaturation of RNase in the presence of Cu(II)

Since values of T_m are the most accurately determined of the thermal denaturation data presented in this thesis, they may be combined with standard thermodynamic expressions to permit the calculation of the enthalpy of denaturation, ΔH^o, without recourse to the van't Hoff method of plotting log K vs 1/T.

At constant pH, the general equation for the effect of temperature on chemical equilibrium applies so long as ΔH^{o} is not a function of the temperature. The equation is

$$\frac{d \ln K}{d\, 1/T} = \frac{- H^{o}}{R} \tag{29}$$

where R is the ideal gas constant. This equation has been shown to apply to the thermal denaturation of RNase by Hermans and Scheraga (1961a). To avoid introducing more than one term in temperature, equation 29 may be integrated indefinitely to give

$$\ln K = \frac{- \Delta H^{o}}{R T_m} + C \tag{30}$$

In the presence of Cu(II) at some concentration at which experimental observations are made, and at unit activity, respectively, one may write

$$\ln K = \frac{- \Delta H^{o}}{RT} + C' \tag{31}$$

and

$$\ln K'' = \frac{- \Delta H^{o}}{RT} + C'' \tag{32}$$

However, the integration constant will vary with Cu(II) and these may be evaluated as

$$C' = \frac{\Delta H^{o}}{R T'_m} \tag{33}$$

and

$$C'' = \frac{\Delta H^{o}}{R T''_m} \tag{34}$$

because the equilibrium constant is equal to unity at the transition temperature. Therefore, if equation 32 is subtracted from equation 31:

$$\ln K - \ln K'' = \frac{-\Delta H^{o}}{RT} - \frac{-\Delta H^{o}}{RT} + C' - C'' \qquad (35)$$

The two identical terms in enthalpy cancel out and substitution from equations 33 and 34 gives

$$\ln K - \ln K'' = \frac{\Delta H^{o}}{RT_m''} - \frac{-\Delta H^{o}}{RT_m'} \qquad (36)$$

Since this derivation holds at constant temperature, it is permissible to substitute from equation 24 to give

$$n \ln [Cu(II)] = \frac{\Delta H^{o}}{T_m''} - \frac{\Delta H^{o}}{T_m'} - n \ln \gamma \qquad (37)$$

Substitution from equation 27 allows

$$n \ln R = \frac{\Delta H^{o}}{T_m''} - \frac{\Delta H^{o}}{T_m'} - n \ln \left\{ [RNase] (1 - \bar{\nu}) \right\} - n \ln \gamma \qquad (38)$$

which may be written as

$$n \ln R = \frac{\Delta H^{o}}{RT_m} + Q' \qquad (12)$$

This equation predicts that a plot of n ln R vs $1/RT_m$ should give a positive linear slope equal to the standard enthalpy of denaturation for RNase.

Equation 12 is subject to all the restrictions in its use as was equation 10, with two exceptions. The restriction on constant temperature is necessarily removed, but an additional restriction is imposed. The cancellation of the terms in enthalpy in equation 35 is valid only if the presence of Cu(II) does not change the value of ΔH^{o} for the thermal denaturation of RNase. Thus equation 12, apart from its use in determining the standard enthalpy, has value in that it provides a test for

the constancy of the enthalpy as R is varied.